Universidade de Aveiro Departamento de Química
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VÂNIA MARIA AMARO CALISTO

OCORRÊNCIA E DESTINO DE FÁRMACOS PSIQUIÁTRICOS NO AMBIENTE

ENVIRONMENTAL OCCURRENCE AND FATE OF PSYCHIATRIC PHARMACEUTICALS

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Química, realizada sob a orientação científica do Doutor Valdemar Inocêncio Esteves, Professor Auxiliar do Departamento de Química da Universidade de Aveiro

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Ao meu marido, Sérgio

o júri

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Prof. Doutor Aníbal Manuel de Oliveira Duarte
professor catedrático da Universidade de Aveiro

Doutor Joaquim Esteves da Silva
professor associado com agregação da Faculdade de Ciências da Universidade do Porto

Doutora Marcela Alves Segundo
professora auxiliar da Faculdade de Farmácia da Universidade do Porto

Doutora Maria do Rosário Gonçalves Reis Marques Domingues
professora auxiliar da Universidade de Aveiro

Doutor Valdemar Inocêncio Esteves
professor auxiliar da Universidade de Aveiro

Doutor Rudolf Josef Schneider
investigador com agregação do BAM Federal Institute for Materials Research and Testing e *lecturer* da Technical University of Berlin, Germany.

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palavras-chave

Fármacos psiquiátricos, carbamazepina, benzodiazepinas, ambiente, persistência, fotodegradação, produtos de fotodegradação, adsorção, electroforese capilar, ELISA, espectrometria de massa.

resumo

Os fármacos são importantes contaminantes ambientais. Nas últimas duas décadas, o número de estudos sobre a ocorrência destes poluentes emergentes em matrizes ambientais aumentou significativamente. Esta ocorrência generalizada preocupa a comunidade científica devido a evidências que comprovam a sua capacidade de interferir nos ecossistemas, mesmo em concentrações muito baixas. No caso particular dos fármacos psiquiátricos é expectável que constituam um risco ecológico significativo. Para uma melhor compreensão do impacto real destes poluentes é essencial que se proceda a uma avaliação extensiva da sua persistência e destino em matrizes ambientais.

Os estudos apresentados nesta tese pretendem contribuir para melhorar o conhecimento acerca da ocorrência, persistência e destino ambiental de fármacos psiquiátricos. Para este efeito, foram seleccionados, como objecto de estudo, dois grupos de fármacos: anti-epilépticos (carbamazepina) e fármacos com efeitos ansiolíticos e sedativos (as benzodiazepinas diazepam, oxazepam, lorazepam e alprazolam).

A fotodegradação é o principal processo que afecta a persistência de poluentes orgânicos em ambientes aquáticos. Consequentemente, a persistência dos cinco fármacos seleccionados foi avaliada através de estudos de fotodegradação directa e indirecta, tendo em consideração a influência de parâmetros relevantes tais como pH, nível de oxigenação e matéria orgânica dissolvida. Os estudos de fotodegradação aqui descritos foram seguidos por cromatografia micelar electrocinética com a aplicação de um capilar com revestimento dinâmico. Adicionalmente, os fotoprodutos resultantes de fotodegradação directa foram identificados por espectrometria de massa.

O estudo da carbamazepina no ambiente é particularmente relevante uma vez que esta foi proposta como um potencial marcador de poluição antropogénica. A sua ocorrência em água superficiais, de sub-solo e residuais foi investigada através da implementação de um ensaio imunológico (ELISA), optimizado para a aplicação a triagens ambientais e amostras com matrizes complexas. O destino deste fármaco na interface água/solo foi também investigado usando solos agrícolas submetidos a fertilizações de longo prazo; este estudo permitiu tirar conclusões acerca da contaminação de águas adjacentes por solos contaminados.

O trabalho aqui descrito constitui uma abordagem multidisplinar à problemática da ocorrência de fármacos psiquiátricos no ambiente, contribuindo de forma relevante para esta área de estudo.

keywords

Psychiatric pharmaceuticals, carbamazepine, benzodiazepines, environment, persistence, photodegradation, photodegradation products, adsorption, capillary electrophoresis, ELISA, mass spectrometry.

abstract

Pharmaceuticals are considered to be important environmental contaminants. During the last two decades, the number of studies reporting the occurrence of these emerging contaminants in a large variety of environmental matrices has undergone a dramatic increase. This widespread occurrence is raising awareness amongst the scientific community due to some evidence indicating their ability to interfere with ecosystems at extremely low concentrations. Psychiatric pharmaceuticals, in particular, are thought to impose significant ecological risks. A better understanding of the real impact of these pollutants implies a comprehensive evaluation of their persistence and fate in environmental matrices.

The studies presented in this thesis aim at providing a significant contribution to increase the existing knowledge related to the occurrence, persistence and environmental fate of psychiatric pharmaceuticals. For this purpose, two groups of psychiatric pharmaceuticals were selected as object of study: anti-epileptics (carbamazepine) and pharmaceuticals with anxiolytic and sedative effects (the benzodiazepines diazepam, oxazepam, lorazepam and alprazolam).

Photodegradation is considered to be the main process affecting the persistence of organic pollutants in aquatic environments. Consequently, the persistence of the five selected psychiatric pharmaceuticals was evaluated by performing direct and indirect photodegradation studies and considering the influence of some relevant parameters such as pH, oxygenation level and dissolved organic matter. The photodegradation studies hereby reported were followed by a newly developed micellar electrokinetic chromatography method, using a dynamically coated capillary. In addition, the photoproducts generated under direct photodegradation were identified by mass spectrometry.

The study of carbamazepine in the environment is particularly relevant as this compound has been recently proposed as a possible marker of anthropogenic pollution. The occurrence of carbamazepine in surface, ground and wastewaters was investigated through the implementation of an immunoassay (ELISA), optimized to perform high throughput environmental screenings in complex matrices. Moreover, the fate of this pharmaceutical at the water/soil interface was studied using agricultural soils submitted to different long-term amendments, allowing for some conclusions on the possible contamination of adjacent water resources by contaminated soils.

The work here presented constitutes a multidisciplinary approach to the environmental occurrence of psychiatric pharmaceuticals and gives a fair contribution to this area of concern.

CONTENTS

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

CHAPTER 2

PSYCHIATRIC PHARMACEUTICALS IN THE ENVIRONMENT: LITERATURE REVIEW

CHAPTER 3

DIRECT PHOTODEGRADATION OF THE ANTI-EPILEPTIC CARBAMAZEPINE

CHAPTER 4

PHOTODEGRADATION OF BENZODIAZEPINES IN AQUATIC ENVIRONMENTS

CHAPTER 5

QUANTIFICATION OF CARBAMAZEPINE IN THE ENVIRONMENT BY ELISA

CHAPTER 6

ADSORPTION OF CARBAMAZEPINE ONTO AGRICULTURAL SOILS

LIST OF TABLES

CHAPTER 2

PSYCHIATRIC PHARMACEUTICALS IN THE ENVIRONMENT: LITERATURE REVIEW

CHAPTER 3

DIRECT PHOTODEGRADATION OF THE ANTI-EPILEPTIC CARBAMAZEPINE

CHAPTER 4

PHOTODEGRADATION OF BENZODIAZEPINES IN AQUATIC ENVIRONMENTS

CHAPTER 5

QUANTIFICATION OF CARBAMAZEPINE IN THE ENVIRONMENT BY ELISA

CHAPTER 6

ADSORPTION OF CARBAMAZEPINE ONTO AGRICULTURAL SOILS

ABBREVIATIONS

ALP – Alprazolam

APCI – Atmospheric pressure chemical ionization

BSA – Bovine serum albumin

CBZ – Carbamazepine

COM – Soil subjected to compost fertilization

CPMAS – Cross polarization magic angle spinning

CR – Cross-reactivity

CTRZ – Cetirizine

CZE – Capillary zone electrophoresis

DBE – Double bond equivalents

DMA – Dimethylacetamide

DOM – Dissolved organic matter

DZP – Diazepam

EC_{50} – Half maximal effective concentration

ELISA – Enzyme-linked immunosorbent assay

EOF – Electroosmotic flow

ESI – Electrospray ionization

GC – Gas chromatography

IC_x – Inhibitory concentration resulting in a decrease in growth by *x*% (chapter 2)

IC_{50} – Concentration resulting in 50% inhibition of the maximum signal (chapter 5)

IgG – Immunoglobulin G

IS – Internal standard

LC – Liquid chromatography

LC_{50} – Concentration resulting in the death of 50% of the population

LLE – Liquid-liquid extraction

LOD – Limit of detection

LOEC – Lowest observable effects concentration

LOQ – Limit of quantification

LRZ – Lorazepam

MEKC – Micellar electrokinetic chromatography

MIN – Soil subjected to mineral fertilization

MRM – Multiple-reaction monitoring mode

MS – Mass spectrometry

M_W – Molecular weight

NMR – Nuclear magnetic resonance

NOEC – No observable effects concentration

OXZ – Oxazepam

PLE – Pressurized liquid extraction

SDS – Sodium dodecylsulphate

SLU – Soil subjected to sewage sludge fertilization

SPE – Solid-phase extraction

SSD – Summer sunny day

SSNRI – Selective serotonin and norepinephrine reuptake inhibitor

SSRI – Selective serotonin reuptake inhibitor

TBABH – Tetrabutylammonium borohydride

TMB – 3,3',5,5'-Tetramethylbenzidine

TOC – Total organic carbon

TOM – Total organic matter

UHPLC – Ultra high-performance liquid chromatography

UPLC – Ultra-performance liquid chromatography

USE – Ultrasonic solvent extraction

UVSD – Ultra-violet spectral deconvolution

WWTP – Wastewater treatment plant

SYMBOLS

C_e – Analyte concentration in the aqueous phase

D_{ow} – pH dependent octanol-water partition coefficient

E – Electric field strength

I^{abs} – Rate of light absorption

I^0 – Lamp emission intensity

k – Pseudo first-order rate constant

K_d – Linear distribution coefficient

K_d^* - Concentration specific distribution coefficient

K_F – Freundlich sorption coefficient

K_L – Langmuir affinity coefficient

K_{ow} – Octanol-water partition coefficient

m_t – Migration time

N – Non-linearity parameter

Q_e – Analyte concentration in the solid phase

q – Charge

r – Correlation coefficient

S – Screening factor

$\mathbf{S}_\lambda$ - Wavelength specific screening factor

$\mathbf{t}_{1/2}$ – Photodegradation half-life time

ϕ – Quantum yield

ϕ_{ave} – Average quantum yield

λ – Wavelength

ε – Molar absorptivity

v_{eo} – Electroosmotic flow velocity

v_{ep} – Migration velocity

ζ – Zeta potential

μ_{ep} – Electrophoretic mobility

σ – Standard deviation

$s_{x/y}$ – Residual standard deviation of the linear regression

η – Viscosity

α_λ – Wavelength specific attenuation coefficient

CHAPTER 1

Introduction

Nowadays, pharmaceuticals are considered to be an important group of environmental contaminants. These compounds are being introduced into the environment on a continuous basis, mainly through wastewater treatment plants, as a result of the inadequacy of the treatment processes applied in these facilities. The occurrence of these widely consumed compounds in a large variety of environmental matrices, as well as the first studies indicating their high persistence and toxicity to non-target organisms, justify the growing concern about these relatively recent environmental pollutants. However, there is still a considerable lack of knowledge about the environmental effects, fate and persistence of these compounds, especially regarding the group of psychiatric pharmaceuticals. This introductory chapter presents a brief overview about pharmaceuticals as environmental contaminants and delineates the main goals of the work presented in this thesis.

CONTENTS

1.1 PHARMACEUTICALS IN THE ENVIRONMENT AN EMERGING CONCERN

Pharmaceuticals are a large and diverse group of organic compounds used in very high amounts throughout the world (Jones et al., 2001; Bound and Voulvoulis, 2004; Jones et al., 2004; Petrovic and Barceló, 2007), developed with the aim of exerting a biological effect (Jones et al., 2005). These compounds are used by humans and other animals (pets and live stock) to treat a large spectrum of diseases, fight infections and reduce disease´s symptoms. Consequently, mainly due to the beneficial effect produced in humans, and also to the increase of life expectancy, the use of pharmaceuticals is expected to grow (Daughton and Ternes, 1999). Nowadays, in the European Union, there are more than 3 000 active substances available in the market (Redshaw et al., 2008).

The first known reports specifically referring to the incomplete removal of some pharmaceuticals by wastewater treatments and the consequent discharge into the environment by wastewater treatment plants (WWTP) were published in the 1960s and 1970s (Stumm-Zollinger and Fair, 1965; Hignite and Azarnoff, 1977). Despite these first findings indicating pharmaceuticals as a potential group of environmental contaminants, this issue did not attract significant attention until the 1990s (Purdom et al., 1994; Desbrow et al., 1998; Routledge et al., 1998) when it was discovered that some of these compounds have the ability to interfere with ecosystems in concentrations as low as a few nanograms per liter (Halling-Sorensen et al., 1998). It was also during that decade that the first optimized analytical methods for the quantification of pharmaceuticals in environmental samples were developed, allowing the determination of very small quantities in aquatic environments (Eckel, 1993; Holm et al., 1995; Ternes, 1998; Ternes et al., 1998; Snyder et al., 1999). Since then, as a consequence of several published studies reporting the occurrence of these compounds in the environment (Ternes, 1998; Ternes et al., 2001; Kolpin et al., 2002; Al-Rifai et al., 2007; Conley et al., 2008), as well as the large amount of pharmaceuticals produced and increasing use and diversity (Bound and Voulvoulis, 2004), the existence of pharmaceutical drugs and pharmaceutically active metabolites in the environment has been considered one of the emerging concerns in environmental sciences (Halling-Sorensen et al., 1998; Daughton and Ternes, 1999; Heberer, 2002; Carlsson et al., 2006). Moreover, and taking into consideration that pharmaceuticals do not occur in the environment individually but as complex mixtures, several investigations showed that toxicity of pharmaceuticals to non-target organisms may be occurring at very low concentrations due to combined and synergistic effects (Henry and Black, 2007; Pomati et al., 2007; DeLorenzo and Fleming, 2008; Pomati et al., 2008; Painter et al., 2009; Quinn et al., 2009; Schnell et al., 2009). These findings were a clear contribution to raise

awareness about this subject and pharmaceuticals are now unanimously considered as an important group of environmental contaminants.

In January 2011, the U.S. Environmental Protection Agency published a list of 11 454 references considered to be directly or peripherally connected to research involving pharmaceuticals and personal care products in the environment (EPA, 2011). Despite the growing number of studies on this subject, there is still much to be understood about environmental transformations, fate (Loffler et al., 2005; Kwon and Armbrust, 2006; Redshaw et al., 2008) and effects of these compounds (Calleja et al., 1994; Brooks et al., 2003a; Brooks et al., 2003b; Brain et al., 2004; Johnson et al., 2007; Gaworecki and Klaine, 2008; Gust et al., 2009).

1.2 SOURCES OF PHARMACEUTICALS INTO THE ENVIRONMENT

Pharmaceuticals are not completely metabolized by the human body and are excreted as the unchanged parent compound or as metabolites or conjugates (generally glucuronides) (Heberer, 2002) (Figure 1.1). Even if pharmaceuticals are extensively metabolized, their metabolites may continue to be biologically active or, in some cases, be easily transformed into the parent compound by hydrolysis or due to bacterial action (Halling-Sorensen et al., 1998; Richards and Cole, 2006). Thus, significant amounts of the parent compound in the unmetabolized form or as metabolites are continuously excreted into the sewage systems, reaching WWTPs. As a result of the inadequacy of removal treatment methods applied in these facilities, the discharge of WWTPs effluents is considered to be the primary pathway of pharmaceuticals into the environment (Kinney et al., 2006a; Gómez et al., 2007; Conkle et al., 2008; Zhang et al., 2008; Loganathan et al., 2009; Jelic et al., 2011). These compounds can also reach aquatic and terrestrial environments due to direct release of treated effluents from production industries, use of treated wastewater for irrigation, land application of sludge, use in veterinary treatments (Pedersen et al., 2005; Kinney et al., 2006a; Kinney et al., 2006b; Ruhoy and Daughton, 2008) and incorrect household disposal of unused packages via trash or sewage (Bound et al., 2006; Ruhoy and Daughton, 2008; Daughton and Ruhoy, 2009). The main sources of pharmaceuticals in the environment and the interconnection between different environmental compartments are illustrated in Figure 1.2. In 2005, Bound and Voulvoulis performed a survey, inquiring approximately 400 householders from the South-East of England, about the disposal and risk perception of pharmaceuticals to the environment. Between 67 and 100 % of the interviewees revealed that usually dispose unused packages into the bin together with the common organic domestic residues and 4 to 17% dispose the drugs into the sink/toilet (Bound and Voulvoulis, 2005). This study, along with more recent

literature, demonstrates that some routes of pharmaceuticals into the environment, considered as secondary, might require greater attention (Daughton and Ruhoy, 2009). The environmental relevance of some secondary sources of pharmaceuticals, as it is the case of incorrect disposal of unused packages, might be reduced by planning and implementing preventive and educational measures.

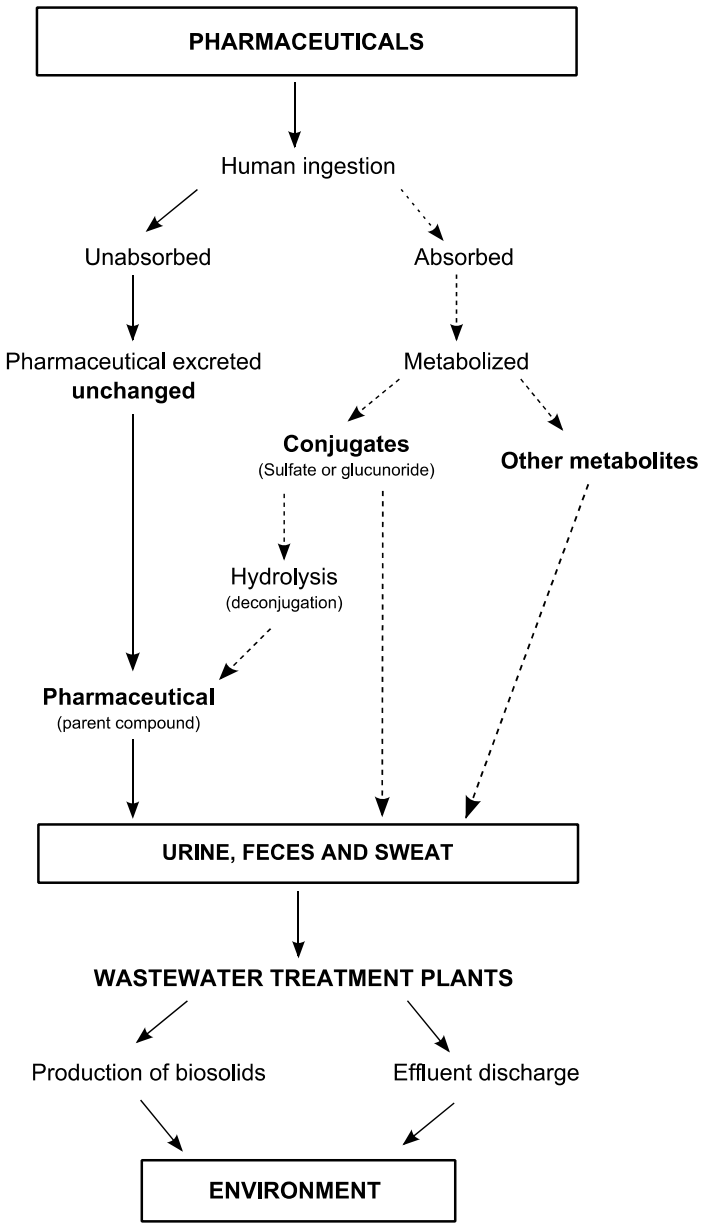

Figure 1.1. Main metabolic processes of pharmaceuticals from human ingestion to excretion into wastewater treatment plants, considered to be the primary source of pharmaceuticals into the environment.

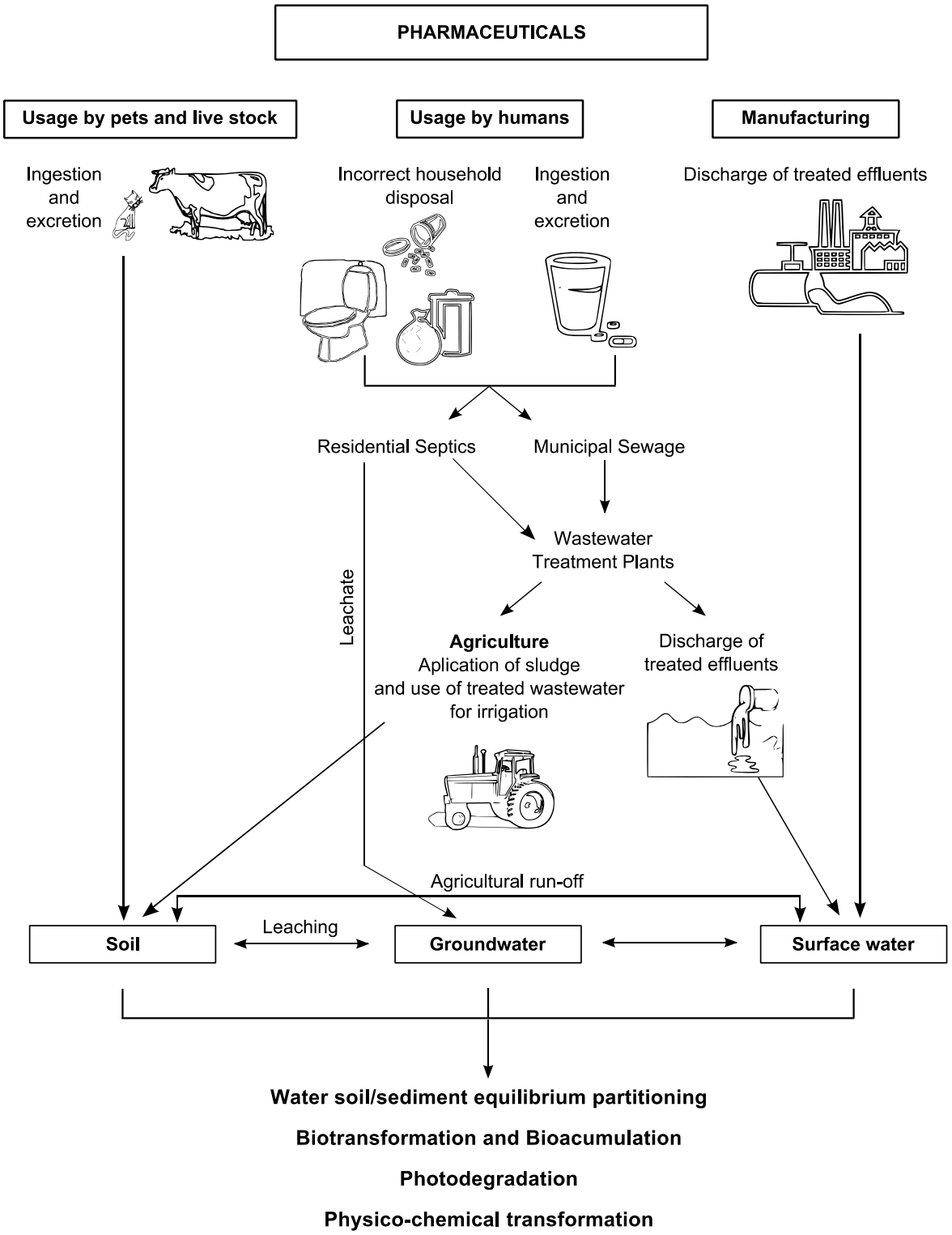

Figure 1.2. Main sources and fate of pharmaceuticals in the environment.

1.3 THE CASE OF PSYCHIATRIC PHARMACEUTICALS

A large diversity of pharmaceuticals has been found in the environment: analgesics, antibiotics, anti-epileptics, β-blockers, blood-lipid regulators, antidepressants, anxiolytics, sedatives, contraceptives, etc. (Jones et al., 2006). Among all the pharmaceutical groups, psychiatric drugs (mainly comprising anxiolytics, sedatives, hypnotics, antidepressants and anticonvulsants) are one of the most commonly prescribed (Schultz and Furlong, 2008). For instance, in North America, in 2009, 18 of the 100 most prescribed active substances were psychoactive pharmaceuticals (Health, 2011). In the specific case of Portugal, and also in 2009, 14 of the most prescribed active substances belong to the pharmacotherapeutic subgroup of psychodrugs with special relevance for benzodiazepines, selective serotonin re-uptake inhibitors (SSRIs) and selective serotonin and norepinephrine re-uptake inhibitors (SSNRIs) (Infarmed, 2009).

In general, pharmaceuticals are used with the intent of having some type of biological or physiological effect in humans or animals. Among their specific characteristics, these compounds have the ability to pass through cellular membranes and are relatively persistent in order not to be inactivated before exerting the desired therapeutic effect (Sanderson et al., 2003; Petrovic and Barceló, 2007). In the particular case of nervous system related pharmaceuticals, and in addition to the referred intrinsic properties, these compounds have great relevance on the regulation of behavior, having the aptitude to directly affect the central nervous system and cause neuro-endocrine signaling disruption. The alteration of the reproduction patterns in non-target aquatic organisms (Brooks et al., 2003b; van der Ven et al., 2004; van der Ven et al., 2006) is one good example that illustrates the possible adverse effects in test organisms, thus reflecting the action mode of this particular group of pharmaceuticals. Several studies have demonstrated that these compounds can affect physiological systems at very low concentrations (van der Ven et al., 2006; Schultz and Furlong, 2008). Van der Ven et al. (2006) showed that mianserin, a tetracyclic antidepressant, has estrogenic activity and produces endocrine disruption in zebrafish. A number of other studies on fluoxetine, diazepam, sertraline, paroxetine, and others, clearly showed significant adverse effects of antidepressants and anxiolytics in living organisms of aquatic matrices (Fong, 1998; Brooks et al., 2003a; Brooks et al., 2003b; Pascoe et al., 2003; Henry et al., 2004; Richards and Cole, 2006; Johnson et al., 2007; Fong and Molnar, 2008; Gaworecki and Klaine, 2008; Gust et al., 2009).

The extremely high rates of consumption of psychiatric pharmaceuticals, in conjunction with their mode of action, reinforce the need to assess contamination levels and better understand

their real ecological impact. However, and when compared to other groups of pharmaceuticals, there is a significant lack of knowledge about psychiatric drugs, especially concerning their dispersion, mobility and persistence under environmental conditions (biotic and abiotic degradability) and the uptake and effects on non-target organisms during chronic and life cycle exposure.

1.4 THESIS LAYOUT

This chapter aimed at presenting a brief overview concerning the occurrence of pharmaceuticals in the environment and some possible consequences to the ecosystems. This complex issue is here only superficially addressed and a more detailed discussion will be the focus of the following chapters.

Amongst the vast universe of pharmaceuticals, psychiatric drugs are thought to pose significant risk to ecosystems and, for this reason, should be made object of special attention. Consequently, the work presented in this thesis was developed with the aim of giving a significant contribution concerning the occurrence, fate and environmental persistence of psychiatric pharmaceuticals.

The work presented in this thesis is divided into five chapters, as follows:

CHAPTER 2 – Literature review. Here, the occurrence, persistence, fate and toxicological relevance of psychiatric pharmaceuticals in the environment are discussed in detail, based on the available literature data. This work resulted in the following publication:

Calisto, V., Esteves, V.I., **2009**. Psychiatric pharmaceuticals in the environment - a review. *Chemosphere* 77, 1257-1274.

CHAPTER 3 – Study of the direct photodegradation of the anti-epileptic pharmaceutical carbamazepine and evaluation of pH and oxygenation levels in the photodegradation rates. This study also includes the development of a micellar electrokinetic chromatography methodology to follow the photodegradation kinetics and identification of photodegradation products by mass spectrometry. This work is published in:

Calisto, V., Domingues, M.R., Erny, G.L., Esteves, V.I., **2011**. Direct photodegradation of carbamazepine followed by micellar electrokinetic chromatography and mass spectrometry. *Water Research* 45, 1095-1104.

CHAPTER 4 – Study of the direct and indirect photodegradation of four benzodiazepines (oxazepam, diazepam, lorazepam and alprazolam), including the effects of different fractions of dissolved organic matter in the photodegradation rates. Similarly to the previous chapter, identification of direct photodegradation products of the studied benzodiazepines by mass spectrometry is also presented. This work is published in:

Calisto, V., Domingues, M.R., Esteves, V.I., **2011** Photodegradation of psychiatric pharmaceuticals in aquatic environments - kinetics and photodegradation products. *Water Research, in press.* doi:10.1016/j.watres.2011.09.008.

CHAPTER 5 – Determination of carbamazepine in ground, surface and wastewaters, collected in Aveiro, Portugal, by an enzyme-linked immunosorbent assay (ELISA) and validation by LC-MS/MS. This research work also involved the optimization of an ELISA for the quantification of carbamazepine in environmental samples with high salinity and high concentrations of dissolved organic matter. This study resulted in the following publication:

Calisto, V., Bahlmann, A., Schneider, R.J., Esteves, V.I., **2011**. Application of an ELISA to the quantification of carbamazepine in ground, surface and wastewaters and validation with LC-MS/MS. *Chemosphere* 84, 1708-1715.

CHAPTER 6 – Evaluation of the fate of carbamazepine at water/soil interfaces by studying the adsorption behavior of this compound onto agricultural soils. Adsorption studies were followed by UV spectral deconvolution and the performance of this recently developed methodology was compared with micellar electrokinetic chromatography.

1.5 REFERENCES

Al-Rifai, J.H., Gabefish, C.L., Schafer, A.I., **2007**. Occurrence of pharmaceutically active and non-steroidal estrogenic compounds in three different wastewater recycling schemes in Australia. *Chemosphere* 69, 803-815.

Bound, J.P., Kitsou, K., Voulvoulis, N., **2006**. Household disposal of pharmaceuticals and perception of risk to the environment. *Environ. Toxicol. Phar.* 21, 301-307.

Bound, J.P., Voulvoulis, N., **2004**. Pharmaceuticals in the aquatic environment – a comparison of risk assessment strategies. *Chemosphere* 56, 1143-1155.

Bound, J.P., Voulvoulis, N., **2005**. Household disposal of pharmaceuticals as a pathway for aquatic contamination in the United Kingdom. *Environ. Health Persp.* 113, 1705-1711.

Brain, R.A., Johnson, D.J., Richards, S.M., Sanderson, H., Sibley, P.K., Solomon, K.R., **2004**. Effects of 25 pharmaceutical compounds to *Lemna gibba* using a seven-day static-renewal test. *Environ. Toxicol. Chem*. 23, 371-382.

Brooks, B.W., Foran, C.M., Richards, S.M., Weston, J., Turner, P.K., Stanley, J.K., Solomon, K.R., Slattery, M., La Point, T.W., **2003**a. Aquatic ecotoxicology of fluoxetine. *Toxicol. Lett.* 142, 169-183.

Brooks, B.W., Turner, P.K., Stanley, J.K., Weston, J.J., Glidewell, E.A., Foran, C.M., Slattery, M., La Point, T.W., Huggett, D.B., **2003**b. Waterborne and sediment toxicity of fluoxetine to select organisms. *Chemosphere* 52, 135-142.

Calleja, M.C., Persoone, G., Geladi, P., **1994**. Comparative acute toxicity of the first 50 multicenter evaluation of in-vitro cytotoxicity chemicals to aquatic non-vertebrates. *Arch. Environ. Con. Tox.* 26, 69-78.

Carlsson, C., Johansson, A.K., Alvan, G., Bergman, K., Kuhler, T., **2006**. Are pharmaceuticals potent environmental pollutants? Part I: environmental risk assessments of selected active pharmaceutical ingredients. *Sci. Total Environ.* 364, 67-87.

Conkle, J.L., White, J.R., Metcalfe, C.D., **2008**. Reduction of pharmaceutically active compounds by a lagoon wetland wastewater treatment system in Southeast Louisiana. *Chemosphere* 73, 1741-1748.

Conley, J.M., Symes, S.J., Kindelberger, S.A., Richards, S.A., **2008**. Rapid liquid chromatography-tandem mass spectrometry method for the determination of a broad mixture of pharmaceuticals in surface water. *J. Chromatogr. A* 1185, 206-215.

Daughton, C.G., Ruhoy, I.S., **2009**. Environmental footprint of pharmaceuticals: The significance of factors beyond direct excretion to sewers. *Environ. Toxicol. Chem.* 28, 2495-2521.

Daughton, C.G., Ternes, T.A., **1999**. Pharmaceuticals and personal care products in the environment: Agents of subtle change? *Environ. Health Persp.* 107, 907-938.

DeLorenzo, M.E., Fleming, J., **2008**. Individual and mixture effects of selected pharmaceuticals and personal care products on the marine phytoplankton species *Dunaliella tertiolecta*. *Arch. Environ. Con. Tox.* 54, 203-210.

Desbrow, C., Routledge, E.J., Brighty, G.C., Sumpter, J.P., Waldock, M., **1998**. Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. *Environ. Sci. Technol.* 32, 1549-1558.

Eckel, W.P., Ross, B., and Isensee, R.K, **1993**. Pentobarbital found in groundwater. Groundwater 31, 801-804.

EPA, U.S., **2011**. Pharmaceuticals and Personal Care Products (PPCPs): Bibliographic Database of Relevant Published Literature. U.S. Environmental Protection Agency, Las Vegas, Nevada (a comprehensive database of literature references compiled and maintained by Daughton and Scuderi; first implemented 19 February 2008); http://www.epa.gov/ppcp/lit.html

Fong, P.P., **1998**. Zebra mussel spawning is induced in low concentrations of putative serotonin reuptake inhibitors. *Biol. Bull*. 194, 143-149.

Fong, P.P., Molnar, N., **2008**. Norfluoxetine induces spawning and parturition in estuarine and freshwater bivalves. *Bull. Environ. Contam. Toxicol.*

Gaworecki, K.M., Klaine, S.J., **2008**. Behavioral and biochemical responses of hybrid striped bass during and after fluoxetine exposure. *Aquat. Toxicol.* 88, 207-213.

Gómez, M.J., Martínez Bueno, M.J., Lacorte, S., Fernández-Alba, A.R., Agüera, A., **2007**. Pilot survey monitoring pharmaceuticals and related compounds in a sewage treatment plant located on the Mediterranean coast. *Chemosphere* 66, 993-1002.

Gust, M., Buronfosse, T., Giamberini, L., Ramil, M., Mons, R., Garric, J., **2009**. Effects of fluoxetine on the reproduction of two prosobranch mollusks: *Potamopyrgus antipodarum* and *Valvata piscinalis*. *Environ. Pollut.* 157, 423-429.

Halling-Sorensen, B., Nielsen, S.N., Lanzky, P.F., Ingerslev, F., Holten Lutzhoft, H.C., Jorgensen, S.E., **1998**. Occurrence, fate and effects of pharmaceutical substances in the environment – a review. *Chemosphere* 36, 357-393.

Health, N., **2011**. The internet drug index (the top 200 prescriptions for 2009 by number dispensed). http://www.rxlist.com (Accessed January 2011).

Heberer, T., **2002**. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. *Toxicol. Lett.* 131, 5-17.

Henry, T.B., Black, M.C., **2007**. Mixture and single-substance acute toxicity of selective serotonin reuptake inhibitors in *Ceriodaphnia dubia*. *Environ. Toxicol. Chem.* 26, 1751-1755.

Henry, T.B., Kwon, J.W., Armbrust, K.L., Black, M.C., **2004**. Acute and chronic toxicity of five selective serotonin reuptake inhibitors in *Ceriodaphnia dubia*. *Environ. Toxicol. Chem*. 23, 2229-2233.

Hignite, C., Azarnoff, D.L., **1977**. Drugs and drug metabolites as environmental contaminants - chlorophenoxyisobutyrate and salicylic-acid in sewage water effluent. *Life Sci.* 20, 337-341.

Holm, J.V., Rugge, K., Bjerg, P.L., Christensen, T.H., **1995**. Occurrence and distribution of pharmaceutical organic-compounds in the groundwater downgradient of a Landfill (Grindsted, Denmark). *Environ. Sci. Technol.* 29, 1415-1420.

Infarmed, **2009**. Estatística do Medicamento - Autoridade Nacional do Medicamento e Produtos de Saúde (Portugal); http://www.infarmed.pt/portal/page/portal/INFARMED/PUBLICACOES/TEMATICOS/ESTATISTICA_MEDICAMENTO

Jelic, A., Gros, M., Ginebreda, A., Cespedes-Sánchez, R., Ventura, F., Petrovic, M., Barcelo, D., **2011**. Occurrence, partition and removal of pharmaceuticals in sewage water and sludge during wastewater treatment. *Water Res.* 45, 1165-1176.

Johnson, D.J., Sanderson, H., Brain, R.A., Wilson, C.J., Solomon, K.R., **2007**. Toxicity and hazard of selective serotonin reuptake inhibitor antidepressants fluoxetine, fluvoxamine, and sertraline to algae. *Ecotox. Environ. Safe.* 67, 128-139.

Jones, O.A.H., Voulvoulis, N., Lester, J.N., **2001**. Human pharmaceuticals in the aquatic environment – a review. *Environ. Technol.* 22, 1383-1394.

Jones, O.A.H., Voulvoulis, N., Lester, J.N., **2004**. Potential ecological and human health risks associated with the presence of pharmaceutically active compounds in the aquatic environment. *Cr. Rev. Toxicol.* 34, 335-350.

Jones, O.A.H., Voulvoulis, N., Lester, J.N., **2005**. Human pharmaceuticals in wastewater treatment processes. *Crit. Rev. Env. Sci. Tech.* 35, 401-427.

Jones, O.A.H., Voulvoulis, N., Lester, J.N., **2006**. Partitioning behavior of five pharmaceutical compounds to activated sludge and river sediment. *Arch. Environ. Con. Tox.* 50, 297-305.

Kinney, C.A., Furlong, E.T., Werner, S.L., Cahill, J.D., **2006**a. Presence and distribution of wastewater-derived pharmaceuticals in soil irrigated with reclaimed water. *Environ. Toxicol. Chem.* 25, 317-326.

Kinney, C.A., Furlong, E.T., Zaugg, S.D., Burkhard, M.R., Werner, S.L., Cahill, J.D., Jorgensen, G.R., **2006**b. Survey of organic wastewater contaminants in biosolids destined for land application. *Environ. Sci. Technol.* 40, 7207-7215.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., **2002**. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: A national reconnaissance. *Environ. Sci. Technol.* 36, 1202-1211.

Kwon, J.W., Armbrust, K.L., **2006**. Laboratory persistence and fate of fluoxetine in aquatic environments. *Environ. Toxicol. Chem.* 25, 2561-2568.

Loffler, D., Rombke, J., Meller, M., Ternes, T.A., **2005**. Environmental fate of pharmaceuticals in water/sediment systems. *Environ. Sci. Technol.* 39, 5209-5218.

Loganathan, B., Phillips, M., Mowery, H., Jones-Lepp, T.L., **2009**. Contamination profiles and mass loadings of macrolide antibiotics and illicit drugs from a small urban wastewater treatment plant. *Chemosphere* 75, 70-77.

Painter, M.M., Buerkley, M.A., Julius, M.L., Vajda, A.M., Norris, D.O., Barber, L.B., Furlong, E.T., Schultz, M.M., Schoenfuss, H.L., **2009**. Antidepressants at environmentally relevant concentrations affect predator avoidance behavior of larval fathead minnows (*Pimephales promelas*). *Environ. Toxicol. Chem.* 28, 2677-2684.

Pascoe, D., Karntanut, W., Muller, C.T., **2003**. Do pharmaceuticals affect freshwater invertebrates? A study with the cnidarian *Hydra vulgaris*. *Chemosphere* 51, 521-528.

Pedersen, J.A., Soliman, M., Suffet, I.H., **2005**. Human pharmaceuticals, hormones, and personal care product ingredients in runoff from agricultural fields irrigated with treated wastewater. *J. Ag. Food Chem.* 53, 1625-1632.

Petrovic, M., Barceló, D. (Eds.), **2007**. *Analysis, Fate and Removal of Pharmaceuticals in the Water Cycle*. Elsevier, Amsterdam, The Netherlands.

Pomati, F., Cotsapas, C.J., Castiglioni, S., Zuccato, E., Calamari, D., **2007**. Gene expression profiles in zebrafish (*Danio rerio*) liver cells exposed to a mixture of pharmaceuticals at environmentally relevant concentrations. *Chemosphere* 70, 65-73.

Pomati, F., Orlandi, C., Clerici, M., Luciani, F., Zuccato, E., **2008**. Effects and interactions in an environmentally relevant mixture of pharmaceuticals. *Toxicol. Sci.* 102, 129-137.

Purdom, C.E., Hardiman, P.A., Bye, V.V.J., Eno, N.C., Tyler, C.R., Sumpter, J.P., 1994. Estrogenic effects of effluents from sewage treatment works. *Chem. Ecol.* 8, 275 - 285.

Quinn, B., Gagné, F., Blaise, C., **2009**. Evaluation of the acute, chronic and teratogenic effects of a mixture of eleven pharmaceuticals on the cnidarian, *Hydra attenuata*. *Sci. Total Environ.* 407, 1072-1079.

Redshaw, C.H., Cooke, M.P., Talbot, H.M., McGrath, S., Rowland, S.J., **2008**. Low biodegradability of fluoxetine HCl, diazepam and their human metabolites in sewage sludge-amended soil. *J. Soils Sediments* 8, 217-230.

Richards, S.M., Cole, S.E., **2006**. A toxicity and hazard assessment of fourteen pharmaceuticals to *Xenopus laevis larvae*. *Ecotoxicology* 15, 647-656.

Routledge, E.J., Sheahan, D., Desbrow, C., Brighty, G.C., Waldock, M., Sumpter, J.P., **1998**. Identification of estrogenic chemicals in STW effluent. 2. In vivo responses in trout and roach. *Environ. Sci. Technol.* 32, 1559-1565.

Ruhoy, I.S., Daughton, C.G., **2008**. Beyond the medicine cabinet: An analysis of where and why medications accumulate. *Environ. Int.* 34, 1157-1169.

Sanderson, H., Johnson, D.J., Wilson, C.J., Brain, R.A., Solomon, K.R., **2003**. Probabilistic hazard assessment of environmentally occurring pharmaceuticals toxicity to fish, daphnids and algae by ECOSAR screening. *Toxicol. Lett.* 144, 383-395.

Schnell, S., Bols, N.C., Barata, C., Porte, C., **2009**. Single and combined toxicity of pharmaceuticals and personal care products (PPCPs) on the rainbow trout liver cell line RTL-W1. *Aquat. Toxicol.* 93, 244-252.

Schultz, M.M., Furlong, E.T., **2008**. Trace analysis of antidepressant pharmaceuticals and their select degradates in aquatic matrixes by LC/ESI/MS/MS. *Anal. Chem.* 80, 1756-1762.

Snyder, S.A., Keith, T.L., Verbrugge, D.A., Snyder, E.M., Gross, T.S., Kannan, K., Giesy, J.P., **1999**. Analytical methods for detection of selected estrogenic compounds in aqueous mixtures. *Environ. Sci. Technol.* 33, 2814-2820.

Stumm-Zollinger, E., Fair, G.M., **1965**. Biodegradation of steroid hormones. *J. Water Poll. Control* Fed. 37, 1506-1510.

Ternes, T., Bonerz, M., Schmidt, T., **2001**. Determination of neutral pharmaceuticals in wastewater and rivers by liquid chromatography-electrospray tandem mass spectrometry. *J. Chromatogr. A* 938, 175-185.

Ternes, T.A., **1998**. Occurrence of drugs in German sewage treatment plants and rivers. *Water Res.* 32, 3245-3260.

Ternes, T.A., Hirsch, R., Mueller, J., Haberer, K., **1998**. Methods for the determination of neutral drugs as well as betablockers and beta(2)-sympathomimetics in aqueous matrices using GC/MS and LC/MS/MS. *Fresen. J. Anal. Chem.* 362, 329-340.

van der Ven, K., Keil, D., Moens, L.N., Hummelen, P.V., van Remortel, P., Maras, M., De Coen, W., **2006**. Effects of the antidepressant mianserin in zebrafish: molecular markers of endocrine disruption. *Chemosphere* 65, 1836-1845.

van der Ven, K., Van Dongen, W., Maes, B.U., Esmans, E.L., Blust, R., De Coen, W.M., **2004**. Determination of diazepam in aquatic samples by capillary liquid chromatography-electrospray tandem mass spectrometry. *Chemosphere* 57, 967-973.

Zhang, Y., Geißen, S.-U., Gal, C., **2008**. Carbamazepine and diclofenac: removal in wastewater treatment plants and occurrence in water bodies. *Chemosphere* 73, 1151-1161.

CHAPTER 2

Psychiatric pharmaceuticals in the environment:

Literature Review

This chapter aims to present a review of the existing literature data concerning the environmental impact of psychiatric drugs (anxiolytics, sedatives, hypnotics, anti-epileptics and antidepressants). This literature review constitutes a multidisciplinary approach to the problems and challenges posed by the occurrence of psychiatric pharmaceuticals in the environment. Through this chapter the following issues will be discussed: occurrence of psychiatric pharmaceuticals in different environmental matrices; metabolization of psychiatric pharmaceuticals in the human body; removal efficiencies of these compounds by wastewater treatment plant methods; environmental persistence and resistance to biotic and abiotic degradation processes; chronic and acute toxicity data to aquatic organisms and, finally, optimization of analytical methods for the determination of psychiatric drugs in environmental complex matrices. The topics here discussed allow for a clear identification of the main research needs of this field of interest.

CONTENTS

2.1 CONTEXTUALIZATION

The number of published studies about the occurrence and effects of pharmaceuticals in the environment has registered a significant increase in the last two decades, largely contributing to increase the knowledge about these emerging environmental contaminants. When discussing environmental problems and the risks caused by these compounds, psychiatric pharmaceuticals are particularly relevant due to the global high rates of consumption and specific mode of action (causing nervous central system related effects). However, psychiatric pharmaceuticals only started to be more closely studied in recent years. This chapter aims to present a literature review concerning the environmental impact of this group of pharmaceuticals, allowing the identification of the research needs of this area. The literature data here presented is essentially focused on anxiolytics, sedatives, hypnotics, the anti-epileptic carbamazepine and several classes of antidepressants. The structures of pharmaceuticals representative of these classes are presented in Figure 2.1.

2.2 OCCURRENCE OF PSYCHIATRIC PHARMACEUTICALS IN THE ENVIRONMENT

2.2.1 Anxiolytics, sedatives and hypnotics

Pharmaceuticals with anxiolytic, sedative and hypnotic effects comprise, essentially, benzodiazepines, buspirone (an azapirone), zoplicone (a cyclopyrrolone), zolpidem (an imidazopyridine) and barbiturates (Chouinard et al., 1999; Kar, 2007). Among these, the group of benzodiazepines (with special relevance to diazepam) is the most extensively studied.

Benzodiazepines are one of the most consumed pharmaceuticals (van der Ven et al., 2004; International Narcotics Control Board, 2010). The most recent data account for 2008, when a total of 30 billion S-DDD ("defined daily doses for statistical purposes") of benzodiazepines were manufactured, the highest amount registered until now, and representing an increase of 3 billion S-DDD relatively to 2007. Alprazolam, diazepam, lorazepam and oxazepam are the most relevant substances of this group, corresponding to 46, 24, 13 and 2% of the total manufactured amount, respectively. Europe registered as the continent with the highest total consumption of benzodiazepines (International Narcotics Control Board, 2010). These compounds act on the central nervous system and are mainly indicated to treat anxiety, amnesia and to produce sedation (Kar, 2007; Brunton, 2008); they are also effective anticonvulsants (Brunton, 2008).

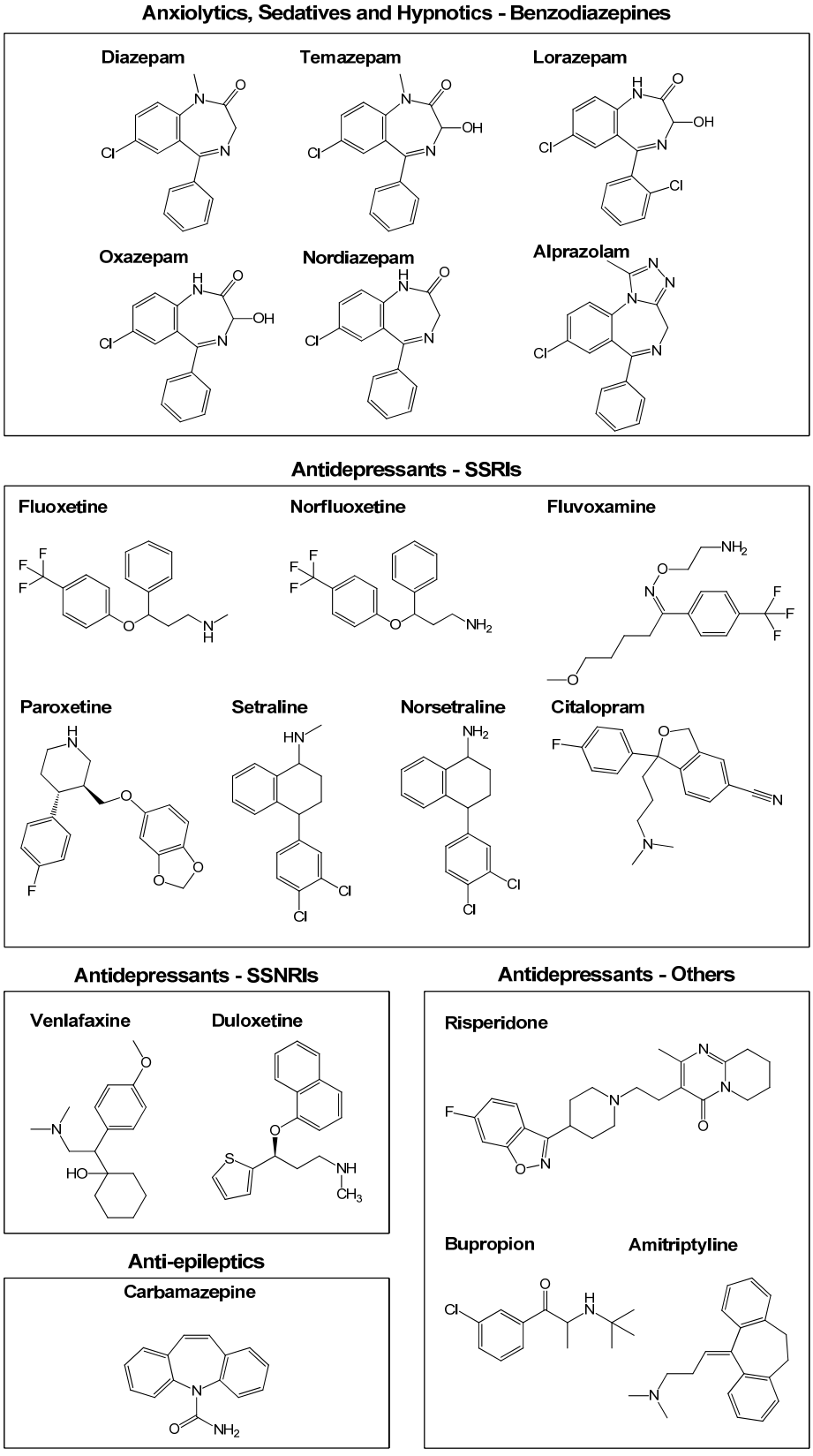

Figure 2.1. Structure of several psychiatric pharmaceuticals.

Nowadays, 35 benzodiazepines are under international control for therapeutic use (International Narcotics Control Board, 2010). However, these compounds are not exclusively used for human therapeutics: benzodiazepines' prescription is also common in veterinary treatments (Courtheyn et al., 2002; Gaskins et al., 2008). Generally, benzodiazepines have anxiolytic and appetite stimulant effects in domestic and wild animals (Courtheyn et al., 2002; Gaskins et al., 2008). Benzodiazepam injections are frequently used to induce anesthesia and diazepam can be used as anxiolytic and sedative in the transportation of sheep, and other domestic animals, to prevent injuries caused by stressful transport conditions.

In Table 2.1 is given an overview of anxiolytics, sedatives and hypnotics found in aquatic environments. Diazepam, a *1,4*-benzodiazepine, is the most studied active substance with anxiolytic effects (Halling-Sorensen et al., 1998; Ternes, 1998; Ternes et al., 2001; Ternes, 2001; Debska et al., 2004; Baker and Kasprzyk-Hordern, 2011). It was first determined in the environment by Waggot (1981) who reported concentrations of < 1 μg L^{-1} in a sewage effluent and ~10 ng L^{-1} in river and potable drinking waters (Halling-Sorensen et al., 1998). Subsequently, several studies indicated the presence of diazepam in WWTPs effluents and influents, rivers and lakes located in distinct parts of the world and even in potable waters. The concentrations found range from 0.49 ng L^{-1}, in untreated drinking water in the U.S. (Benotti et al., 2009), to 1.18 μg L^{-1}, in a WWTP in Belgium (van der Ven et al., 2004). In surface waters, a maximum concentration of 0.88 μg L^{-1} was found, in Germany (Ternes, 2001). Diazepam was also found in fish liver, with concentrations up to 110 ng g^{-1}, in the U.S. (Kwon et al., 2009). Recently, a study on pharmaceutical contaminants and associated potential concerns to pregnant women and children reported that a cumulative ingestion of 5% of a minimum clinical dose of diazepam may occur during pregnancy (taking as reference the ingestion of 2 liters of water per day, during 36 weeks, and a determined diazepam concentration in drinking water of 0.235 μg L^{-1}) (Collier, 2007). In the author's opinion, this fact may not be ignored as there are strong evidences that diazepam might cause several dysfunctions when used in the later stages of pregnancy (such as the withdrawal and the Floppy infant syndrome) (Collier, 2007). The few data available on the occurrence of the large majority of other benzodiazepines are referred, mainly, to the last two years, indicating the recent interest on these compounds (Gracia-Lor et al. 2011; Huerta-Fontela et al., 2010; López-Serna et al., 2010). No data was found concerning buspirone, zolpicone and zolpidem.

The other referred pharmaceutical group with anxiolytic, sedative and hypnotic action is the barbiturates' group which is constituted by derivatives of barbituric acid (Peschka et al., 2006). Until the 1970s, barbiturate derivatives were the most common active substances in sleeping pills (Holm et al., 1995) and were also commonly used to treat anxiety symptoms (Kar, 2007). In

addition, this group has an important therapeutic application as narcotics (Peschka et al., 2006). Nevertheless, with the exception of some specific usages, barbiturates have been replaced by benzodiazepines, due to the severe adverse effects to the human body (Kar, 2007; Brunton, 2008) and, nowadays, are mainly used as veterinary drugs (Peschka et al., 2006). Despite the small number of barbiturate´s prescriptions to human therapeutics, it is relevant to refer that some studies reported the occurrence of these compounds in the environment. An investigation performed by Eckel (1993) determined pentobarbital in groundwater near a landfill in Florida that used to receive medical wastes in 1968 and 1969, thus demonstrating that this pharmaceutical had persisted in the environment during 21 years (Eckel, 1993). A distinct research done by Holm et al. (1995) revealed a similar situation with *5,5*-diallybarbituric acid. Holm analyzed groundwater close to a landfill, in Denmark, which received, approximately, 85 000 tones of industrial and domestic wastes between 1962 and 1975. Once more, the barbiturate was identified in the analyzed samples, indicating an environmental persistence of more than 20 years (Holm et al., 1995). More recent studies reported concentrations of barbiturates up to 5.4 μg L^{-1} in surface waters, in Germany (Peschka et al., 2006).

2.2.2 Anti-epileptics - Carbamazepine

Carbamazepine, a dibenzazepine, is the most representative compound of the anti-epileptic pharmaceuticals. It has anticonvulsant properties and is used in the treatment of psychomotor epilepsy, grand mal (and seizures, in general) and it is also crucial for the relief of pain caused by trigeminal neuralgia (Kar, 2007). It is estimated that 1 014 tons of carbamazepine are consumed worldwide, per year (Zhang et al., 2008).

This pharmaceutical is one of the most frequently detected in the environment, with numerous studies reporting its occurrence all over the world. Some relevant data about its presence in WWTPs effluent, influents, surface and ground waters are summarized in Table 2.2. Concentrations found in the environment range from 3.3 ng L^{-1} in surface waters, France (Togola and Budzinski, 2007), to 11.6 μg L^{-1}, also in surface waters, in a European river (Loos et al., 2009). Note that the maximum concentration of carbamazepine reported until now (11.6 μg L^{-1}) is remarkably high, especially when compared to the concentration of other psychiatric pharmaceuticals in surface waters. Recently, a European survey published data of the greatest relevance about contamination levels in Europe, concluding that carbamazepine was found with a frequency of 95% in 122 river water samples, and of 42% in 164 ground water samples (Loos et al., 2009; Loos et al., 2010).

Table 2.1. Occurrence of anxiolytics, sedatives and hypnotics in the environment.

Concentration found in the environment	Sample	Method of analysis	Additional information	Reference
		DIAZEPAM		
<1 μg L^{-1}	Sewage effluent; UK	Not provided	Analysis performed in 1981	(Halling-Sorensen et al., 1998)
~10 ng L^{-1}	River water; UK	Not provided	Analysis performed in 1981	(Halling-Sorensen et al., 1998; Jones et al., 2005a)
~10 ng L^{-1}	Potable water; UK	Not provided	Analysis performed in 1981	(Halling-Sorensen et al., 1998; Jones et al., 2005a)
0.04 μg L^{-1}	Municipal sewage treatment plant (STP) effluents; Germany	GC-MS	Analysis performed between 1996-1998	(Ternes, 1998, 2001)
23.5 ng L^{-1}	Drinking water; Italy	HPLC-MS/MS	-	(Zuccato et al., 2000)
< LOQ (0.20 μg L^{-1})	Influent of a municipal Hessian STP; Germany	LC–ES-MS/MS	Analysis performed between 26/06/00 and 30/06/00	(Ternes et al., 2001)
< LOQ (0.050 μg L^{-1})	Effluent of a municipal Hessian STP; Germany	LC–ES-MS/MS	Analysis performed between 26/06/00 and 30/06/00	(Ternes et al., 2001)
0.053 μg L^{-1}	Municipal STP effluent; Germany	LC–ES-MS/MS	-	(Ternes et al., 2001)
0.033 μg L^{-1}	Rivers and streams; Germany	LC–ES-MS/MS	-	(Ternes et al., 2001)
0.88 μg L^{-1}	Surface waters; Germany	HPLC-MS/MS	Analysis performed in 2000	(Ternes, 2001)
3-62 ng L^{-1}	Lake Mead; USA	GC-MS	Analysis performed between 1997 and 1999	(Snyder et al., 2001)
0.13 – 2.13 ng L^{-1}	Po and Lambro rivers; Italy	HPLC-MS	Diazepam was found in all the 8 samples collected in different locations. Sampling performed in October, 2001.	(Calamari et al., 2003)
>0.01 μg L^{-1}; 0.59 μg L^{-1}; 1.18 μg L^{-1}	STP influent; Belgium	LC-ES–MS/MS	-	(van der Ven et al., 2004)
>0.01 μg L^{-1}; 0.66 μg L^{-1}	STP influent; Belgium	LC-ES–MS/MS	-	(van der Ven et al., 2004)
120 ng L^{-1}	Wastewater sewer; Germany	LC-MS/MS	Sampling between 2001 and 2003	(Leif et al., 2004)
310 ng L^{-1}	Wastewater inflow; WWTP in Germany	LC-MS/MS	Sampling between 2001 and 2003	(Leif et al., 2004)
33.6 ± 7.1; 30.8 ± 9.3 and 27.9 ± 5.1 ng L^{-1}	River water; Romania	GC-MS	-	(Moldovan, 2006)

Concentration found in the environment	Sample	Method of analysis	Additional information	Reference
23 – 45 ng/g, female fish; 58 – 110 ng/g, male fish (wet weight)	Fish liver; USA (California)	LC-MS/MS	Fish species: *Pleuronichthys verticalis*	(Kwon et al., 2009)
0.47 ng L^{-1}	Untreated drinking water, USA	LC-MS/MS	Maximum concentration found	(Benotti et al., 2009)
6.52 (± 0.64%) ng L^{-1}	Llobregat River basin; Spain	LC-MS/MS	Samples collected downstream to WWTP effluent discharge	(López-Serna et al., 2010)
49 ng L^{-1}	6 WWTP effluents; Spain	UPLC-MS/MS	-	(Huerta-Fontela et al., 2010)
1.1 ng L^{-1}	Surface waters; UK	LC-MS/MS	Samples collected in 6 river locations in UK	(Baker and Kasprzyk-Hordern, 2011)
NORDIAZEPAM				
8 ng L^{-1}	WWTP effluent; France	GC-MS	-	(Rabiet et al., 2006)
8.3 ng L^{-1}	WWTP effluent; France	GC-MS	-	(Togola and Budzinski, 2008)
2.4 ng L^{-1}	Surface waters; France	GC-MS	-	(Togola and Budzinski, 2008)
19 – 76 ng L^{-1}	Surface water; Spain	LC-MS	Samples collected in 5 rivers of Madrid	(González Alonso et al., 2010)
5.5 ng L^{-1}	Surface waters; UK	LC-MS/MS	Samples collected in 6 river locations in UK	(Baker and Kasprzyk-Hordern, 2011)
OXAZEPAM				
0.25 µg L^{-1}	STP effluent; Germany	GC-MS	-	(Heberer, 2002)
6 – 129 ng L^{-1}	Surface water; Spain	LC-MS	Samples collected in 5 rivers of Madrid	(González Alonso et al., 2010)
21 – 324 ng L^{-1}	6 WWTP effluents; Spain	UPLC-MS/MS	-	(Huerta-Fontela et al., 2010)
17.4 ng L^{-1}	Surface waters; UK	LC-MS/MS	Samples collected in 6 river locations in UK	(Baker and Kasprzyk-Hordern, 2011)
ALPRAZOLAM				
1 ng L^{-1}	6 WWTP effluents; Spain	UPLC-MS/MS	-	(Heberer, 2002)
7 ng L^{-1}	WWTP effluents; Spain	UPLC-MS/MS	Maximum concentration found	(Gracia-Lor et al., 2011)

Concentration found in the environment	Sample	Method of analysis	Additional information	Reference
		LORAZEPAM		
31 ± 1 – 196 ± 7 ng L^{-1}	STP effluent; France	LC-MS/MS	Samples collected between July 2007 – February 2008	(Coetsier et al., 2009)
22.58 (± 0.14%) and 41.27 (± 0.23%) ng L^{-1}	Llobregat River basin; Spain	LC-MS/MS	Samples collected downstream and upstream to WWTP effluent discharge	(López-Serna et al., 2010)
4 – 532 ng L^{-1}	6 WWTP effluents; Spain	UPLC-MS/MS	-	(Huerta-Fontela et al., 2010)
40 ng L^{-1}	Surface waters; Spain	LC-MS	-	(Gros et al., 2010)
81 ng L^{-1}	WWTP effluents; Spain	UPLC-MS/MS	Maximum concentration found	(Gracia-Lor et al., 2011)
		PENTOBARBITOL		
Qualitative analysis	Groundwater near a landfill; USA	GC-MS/MS	Analysis performed in 1991. The landfill received medical wastes in 1968 and 1969	(Eckel, 1993; Jones et al., 2001)
5.4 µg L^{-1}	River Mulde; Germany	GC-MS	Analysis performed in April 2004	(Peschka et al., 2006)
		BUTALBITAL		
5.3 µg L^{-1}	River Mulde; Germany	GC-MS	Analysis performed in April 2004	(Peschka et al., 2006)
		PHENOBARBITAL		
0.03 µg L^{-1}	STP effluent; Germany	GC-MS	-	(Heberer, 2002)
0.2 – 1.3 µg L^{-1}	Irrigation field; Germany	GC-MS	Analysis performed in 2004/2005	(Peschka et al., 2006)
		5,5-DIALLYLBARBITURIC ACID		
Qualitative analysis	Groundwater near a landfill; Denmark	HPLC-UV	The landfill received ~85 000 tones of pharmaceutical industrial and domestic wastes between 1962-1975.	(Holm et al., 1995)
		MEPROBAMATE		
Qualitative analysis	Groundwater near a landfill; USA	GC-MS/MS	Analysis performed in 1991. The landfill received medical wastes in 1968 and 1969	(Eckel, 1993; Jones et al., 2001)
43 ng L^{-1}	Finished drinking waters; USA	Not provided	-	(Snyder, 2008)
42 – 73 ng L^{-1}	Drinking waters; USA	GC-MS/MS	-	(Benotti et al., 2009)

Table 2.2. Occurrence of the anti-epileptic carbamazepine in the environment.

Concentration found in the environment	Sample	Method of analysis	Additional information	Reference
		CARBAMAZEPINE		
0.3 – 1.2 µg L^{-1}	STP effluents: France, Greece, Italy and Sweden	LC-MS	Samples collected between February and March 2001	(Andreozzi et al., 2003)
157 – 293 ng L^{-1}	STP effluent; South France	GC-MS	-	(Rabiet et al., 2006)
13.9 – 43.2 ng L^{-1}	Wells supplying drinking water, France	GC-MS	-	(Rabiet et al., 2006)
30.9 ± 7.8 – 2519.3 ± 147.6 ng L^{-1}	WWTP effluent, France	GC-MS	Samples collected between March 2002 and November 2002	(Togola and Budzinski, 2007)
3.3 ± 1.3 – 82.7 ± 13.2 ng L^{-1}	Surface water, Seine river estuary, France	GC-MS	Samples collected between March 2002 and November 2002	(Togola and Budzinski, 2007)
326 ± 3 - 1573 ±34 ng L^{-1}	STP effluent; France	LC-MS/MS	Samples collected between July 2007 and February 2008	(Coetsier et al., 2009)
0.54 ± 0.01 – 3.0 ± 0.2 µg L^{-1}	Surface waters; Germany	ELISA and LC-MS/MS	-	(Bahlmann et al., 2009)
2.3 ± 0.1 µg L^{-1}	WWTP effluent; Germany	ELISA and LC-MS/MS	-	(Bahlmann et al., 2009)
11561 ng L^{-1} (Max.); 248 ng L^{-1} (Average)	122 samples of European Rivers	LC-MS/MS	European survey with the participation of 27 countries, with a detection frequency of 95%.	(Loos et al., 2009)
178 ng L^{-1}	Douro river, Portugal	LC-MS/MS	Samples collected downstream from WWTP effluent discharge	(Madureira et al., 2009)
51 ng L^{-1}	Untreated drinking water; USA	LC-MS/MS	Maximum concentration found	(Benotti et al., 2009)
31.28 (± 0.29%) and 58.43 (± 0.30%) ng L^{-1}	Llobregat River basin; Spain	LC-MS/MS	Samples collected downstream and upstream from WWTP effluent discharge	(López-Serna et al., 2010)
35 – 1160 ng L^{-1}	Surface water; Spain	LC-MS	Samples collected in 5 rivers of Madrid	(González Alonso et al., 2010)
5 – 175 ng L^{-1}	6 WWTP effluents; Spain	UPLC-MS/MS	-	(Huerta-Fontela et al., 2010)
390 ng L^{-1} (Max.); 12 ng L^{-1} (Average)	164 samples of ground water, Europe	LC-MS/MS	European survey with the participation of 23 countries, with a detection frequency of 42%.	(Loos et al., 2010)
0.145 µg L^{-1}	Ter River; Spain	GC-ECD	-	(Calderón-Preciado et al., 2011)

2.2.3 Antidepressants

Antidepressants are a largely prescribed group of pharmaceuticals (Kwon and Armbrust, 2008; Paterson and Metcalfe, 2008; Health, 2011). One of the most common classes of antidepressants is known as selective serotonin reuptake inhibitors (SSRIs) and act by modulating the levels of the neurotransmitter serotonin (Brooks et al., 2005). They are largely prescribed to treat clinical depression, compulsive-obsessive disorder, panic disorder, as well as other cases in which selective inhibition of serotonin reuptake is desirable (Brooks et al., 2003a; Schultz and Furlong, 2008; Unceta et al., 2008). Presently, there are five SSRIs available in the market: fluoxetine, fluvoxamine, paroxetine, sertraline and citalopram (Johnson et al., 2007). There are other antidepressants usually prescribed when SSRIs are not effective. These include venlafaxine and duloxetine (selective serotonin and norepinephrine reuptake inhibitors (SSNRIs)), bupropion (that acts by inhibiting the uptake of dopamine and norepinephrine) (Schultz and Furlong, 2008) and also tricyclic and tetracyclic antidepressants such as amitriptyline and mianserin, respectively. Some of these, and particularly amitriptyline, are also administered to animals (Halling-Sorensen et al., 1998). The usage of antidepressants in animals is recommended to treat pathologies such as separation anxiety, obsessive-compulsive and fearful behaviors amongst other behavioral problems (Mills, 2003).

A large number of antidepressants has already been identified in water, sludge and biological tissues of aquatic organisms and, as for the case of anxiolytics, sedatives and hypnotics, concentrations found range from ng to μg L^{-1} or ng to μg kg^{-1} (Kolpin et al., 2002; Metcalfe et al., 2003; Brooks et al., 2005; Kinney et al., 2006; Schultz and Furlong, 2008). Detailed information about the environmental occurrence of antidepressants is presented in Table 2.3. Fluoxetine (a SSRI) and its metabolite norfluoxetine (biologically active and more potent than the parent compound) are the most commonly investigated antidepressants throughout the world. However, the antidepressants found in the highest concentrations were venlafaxine, citalopram and bupropion (1000 ± 400 ng L^{-1}, 90 ± 20 ng L^{-1}, 60 ± 40 ng L^{-1}, respectively, in samples collected downstream from a water reclamation plant) (Schultz and Furlong, 2008). Until now, the maximum determined concentration of fluoxetine was 0.099 μg L^{-1} in WWTP effluents in Canada (Metcalfe et al., 2003). Moreover, very high quantities of fluoxetine were found in biosolids produced by a WWTP, varying from 100 to 4700 μg $kg_{organic\ carbon}^{-1}$ (Kinney et al., 2006). The determination of fluoxetine concentration in biosolids may be a helpful tool to understand the probable environmental fate of fluoxetine in water/sediment systems and also to identify the usage of sludge in agricultural fields as a significant pathway to the entrance of this compound into the environment (Xia et al., 2005), as it was previously referred in chapter 1.

Table 2.3. Occurrence of antidepressants in the environment.

Concentration found in the environment	Sample	Method of analysis	Additional information	Reference
		FLUOXETINE		
0.012 μg L^{-1}	Surface waters; USA	LC- (ESI(+))-MS	Analysis performed between 1999 and 2000	(Kolpin et al., 2002)
0.099 μg L^{-1}	Effluents of sewage treatment plants; Canada	GC-MS	-	(Metcalfe et al., 2003)
0.1 ng g^{-1} – 10 ng g^{-1}	Tissues (muscle, brain and liver) of fish living in a municipal effluent-dominated stream; USA	GC-MS	Fish species: *Lepomis macrochirus, Ictalurus punctatus, Cyprinus carpioand, Pomoxis nigromaculatus*	(Brooks et al., 2005)
100 – 4700 μg kg^{-1} organic carbon	9 biosolids produced by 8 WWTPs; USA	HPLC-(ESI)-MS	-	(Kinney et al., 2006)
0.14 – 1.02 μg kg^{-1}	Fish tissues; Canada	LC-(APCI)-MS/MS	Fish species: *Ameiurus nebulosus*, *Dorosoma cepedianum* and *Morone americana*	(Chu and Metcalfe, 2007)
12 ± 3; 20 ± 10 and 12 ± 5 ng L^{-1}	5 to 1762 m of distance downstream from the Pecan Creek Water Reclamation Plant; USA	LC-(ESI)-MS/MS	-	(Schultz and Furlong, 2008)
< 0.00050 μg L^{-1}	Finished drinking water; USA	Not provided	-	(Snyder, 2008)
3.0 ng L^{-1}	Untreated drinking water; USA	LC-MS/MS	Maximum concentration found	(Benotti et al., 2009)
2.74 ng L^{-1} (± 2.24%)	Drinking water; Spain	LC-MS/MS	-	(López-Serna et al., 2010)
15.87 ng L^{-1} (± 0.25%)	WWTP effluent, Llobregat River basin; Spain	LC-MS/MS	-	(López-Serna et al., 2010)
8 – 44 ng L^{-1}	Surface water; Spain	LC-MS	Samples collected in 5 rivers of Madrid	(González Alonso et al., 2010)
13.5 ng L^{-1}	Surface water; UK	LC-MS/MS	Samples collected in 6 river locations in UK	(Baker and Kasprzyk-Hordern, 2011)
		NORFLUOXETINE		
0.1 ng g^{-1} - 10 ng g^{-1}	Tissues (muscle, brain and liver) of fish living in a municipal effluent-dominated stream; USA	GC-MS	Fish species: *Lepomis macrochirus, Ictalurus punctatus, Cyprinus carpioand Pomoxis nigromaculatus*	(Brooks et al., 2005)
0.15 – 1.08 μg kg^{-1}	Fish tissues; Canada	LC-(APCI)-MS/MS	Fish species: *Ameiurus nebulosus*, *Dorosoma cepedianum* and *Morone americana*	(Chu and Metcalfe, 2007)

Concentration found in the environment	Sample	Method of analysis	Additional information	Reference
0.83 ± 0.01; 1.0 ± 0.5 and 0.9 ± 0.2 ng L^{-1}	5 to 1762 m of distance downstream from the Pecan Creek Water Reclamation Plant; USA	LC-(ESI)-MS/MS	-	(Schultz and Furlong, 2008)
		PAROXETINE		
0.48 – 0.58 µg kg^{-1}	Fish tissues; Canada	LC-(APCI)-MS/MS	Fish species: *Ameiurus nebulosus*, *Dorosoma cepedianum* and *Morone americana*	(Chu and Metcalfe, 2007)
2.1 ± 0.4; 3 ± 1 and 2.2 ± 0.2 ng L^{-1}	5 to 1762 m of distance downstream from the Pecan Creek Water Reclamation Plant; USA	LC-(ESI)-MS/MS	-	(Schultz and Furlong, 2008)
		SERTRALINE		
0.1 ng g^{-1} - 10 ng g^{-1}	Tissues (muscle, brain and liver) of fish living in a municipal effluent-dominated stream; USA	GC-MS	Fish species: *Lepomis macrochirus, Ictalurus punctatus, Cyprinus carpioand Pomoxis nigromaculatus*	(Brooks et al., 2005)
36 ± 5; 49 ± 9 and 33 ± 8 ng L^{-1}	5 to 1762 m of distance downstream from the Pecan Creek Water Reclamation Plant; USA	LC-(ESI)-MS/MS	-	(Schultz and Furlong, 2008)
		NORSERTRALINE		
0.1 ng g^{-1} – 10 ng g^{-1}	Tissues (muscle, brain and liver) of fish living in a municipal effluent-dominated stream; USA.	GC-MS	Fish species: *Lepomis macrochirus, Ictalurus punctatus, Cyprinus carpioand Pomoxis nigromaculatus*	(Brooks et al., 2005)
5 ± 3; 7 ± 3 and 3 ± 1 ng L^{-1}	5 to 1762 m of distance downstream from the Pecan Creek Water Reclamation Plant; USA	LC-(ESI)-MS/MS	-	(Schultz and Furlong, 2008)
		CITALOPRAM		
90 ± 20; 40 ± 30 and 80 ± 30 ng L^{-1}	5 to 1762 m of distance downstream from the Pecan Creek Water Reclamation Plant; USA	LC-(ESI)-MS/MS	-	(Schultz and Furlong, 2008)
3 – 120 ng L^{-1}	Surface water; Spain	LC-MS	Samples collected in 5 rivers of Madrid	(González Alonso et al., 2010)

Concentration found in the environment	Sample	Method of analysis	Additional information	Reference
		DULOXETINE		
1.5 ± 0.2; 2 ± 2 and 1.2 ± 0.9 ng L^{-1}	5 to 1762 m of distance downstream from the Pecan Creek Water Reclamation Plant; USA	LC-(ESI)-MS/MS	-	(Schultz and Furlong, 2008)
		BUPROPION		
50 ± 20; 60 ± 40 and 50 ± 10 ng L^{-1}	5 to 1762 m of distance downstream from the Pecan Creek Water Reclamation Plant; USA	LC-(ESI)-MS/MS	-	(Schultz and Furlong, 2008)
		VENLAFAXINE		
600 ± 200; 1000 ± 400 and 900 ± 300 ng L^{-1}	5 to 1762 m of distance downstream from the Pecan Creek Water Reclamation Plant; USA	LC-(ESI)-MS/MS	-	(Schultz and Furlong, 2008)
22 – 387 ng L^{-1}	Surface water; Spain	LC-MS	Samples collected in 5 rivers of Madrid	(González Alonso et al., 2010)
875 ng L^{-1}	Surface waters; Spain	UPLC-MS/MS	Maximum concentration found	(Gracia-Lor et al., 2011)
575 ng L^{-1}	Surface waters; Spain	UPLC-MS/MS	Maximum concentration found	(Gracia-Lor et al., 2011)
71.6 ng L^{-1}	Surface water; UK	LC-MS/MS	Samples collected in 6 river locations in UK	(Baker and Kasprzyk-Hordern, 2011)
		RISPERIDONE		
0.00034 μg L^{-1}	Finished drinking water; USA.	Not provided	-	(Snyder, 2008)
		AMITRIPTYLINE		
0.5 – 21 ng L^{-1}; 0.5 – 3 ng L^{-1} ;0.5 – 17 ng L^{-1}; 0.5 – 13 ng L^{-1}	Rivers in South Wales, UK	HPLC-MS/MS	-	(Kasprzyk-Hordern et al., 2008)
6.0 ng L^{-1}	WWTP effluent; France	GC-MS	-	(Togola and Budzinski, 2008)
1.4 ng L^{-1}	Drinking water; France	GC-MS	-	(Togola and Budzinski, 2008)
71.6 ng L^{-1}	Surface water; UK	LC-MS/MS	Samples collected in 6 river locations in UK	(Baker and Kasprzyk-Hordern, 2011)
		LOFEPRAMINE		
<4 ng L^{-1}	Estuaries; UK	LC-(ESI)-MS/MS	-	(Thomas and Hilton, 2004)

Besides the occurrence in surface waters (Thomas and Hilton, 2004; Richards and Cole, 2006; Kasprzyk-Hordern et al., 2008; González Alonso et al., 2010; Baker and Kasprzyk-Hordern, 2011), amitriptyline, fluoxetine and risperidone were also recently found in treated drinking waters in concentrations in the order of ng L^{-1} (Togola and Budzinski, 2008; Benotti et al., 2009; López-Serna et al., 2010), revealing the inefficiency of drinking water treatments to remove these compounds. Additionally, several antidepressants were determined in biological fish tissues, emphasizing the possibility of bioaccumulation by aquatic organisms. Brooks et al. (2005) found concentrations in the range of 0.1 to 10 ng g^{-1} of fluoxetine, sertraline and their metabolites (norfluoxetine and desmethylsertraline, respectively) in muscle, liver and brain tissues of four fish species in a municipal effluent-dominated stream, in Texas, USA. A similar experiment by Chu and Metcalfe (2007) reported concentrations from 0.14 to 1.02 μg kg^{-1} of fluoxetine, 0.15 to 1.08 μg kg^{-1} of norfluoxetine and 0.48 to 0.58 μg kg^{-1} of paroxetine in other four fish species. The implications of these findings, concerning the toxicological relevance of the contamination levels of living organisms, will be discussed later, in section 2.6.

2.3 METABOLIZATION OF PSYCHIATRIC PHARMACEUTICALS

As it was mentioned in chapter 1, pharmaceuticals ingested by humans are almost always not completely metabolized, resulting in the excretion of variable percentages of the active compound along with several metabolites and conjugates in urine and feces (Carlsson et al., 2006). Excretion by patients is considered to be the main pathway for the entrance of pharmaceuticals into the environment (due to the inadequacy of removal methods of WWTPs) (Sanderson et al., 2003; Cunningham, 2006; Jelic et al., 2011) and thus, human metabolism and excretion rates of psychiatric drugs should be addressed.

Psychiatric pharmaceuticals, and in particular benzodiazepines, are usually excreted in urine, being first extensively metabolized in the liver to form pharmacologically inactive glucuronide conjugates (Chouinard et al., 1999). Despite the pharmacological inactivity of these conjugates, it is thought that they are easily decomposed by bacterial action and reconverted in the parent active compound (Halling-Sorensen et al., 1998; Ternes, 2001; Ashton et al., 2004; Carballa et al., 2004). It seems to be highly probable that glucuronide conjugates are readily deconjugated in domestic wastewaters and WWTPs due to the generalized presence of the fecal bacteria *Escherichia coli*, responsible for the production of very large amounts of the enzyme β-glucuridase (Ternes, 1998; Jones et al., 2005b; Petrovic and Barceló, 2007). This deconjugation process results in the increase of the parent compound's quantity in sewage conditions.

For a more detailed analysis of this subject, Table 2.4 presents a compilation of literature data about the excretion rates and most important metabolites of some psychiatric drugs that occur in the environment, based on data presented in Tables 2.1 - 2.3, section 2.2. Large discrepancies of excretion rates are found in the literature. The amounts of excreted pharmaceuticals in the unchanged or conjugate forms can vary considerably, from 1 to 80% of a single dose. The benzodiazepines alprazolam and oxazepam are examples of pharmaceuticals with excretion rates of 75 to 80%, in unchanged or conjugate forms. Moreover, some pharmaceuticals are released as pharmacologically active metabolites, as it is the case of tetrazepam which, until now, is not reported to occur in the environment but is excreted as diazepam (from 13 to 49%).

Table 2.4. Metabolites and excretion rates of psychiatric pharmaceuticals which occur in the environment.

Excretion rates	Additional information
DIAZEPAM	
10% unchanged (Carballa et al., 2008) 1% unchanged (Smith-Kielland et al., 2001) Conjugated metabolites can be 22-43% of a single intake dose (Smith-Kielland et al., 2001) <5% of the parent compound excreted (Jjemba, 2006) Mean amounts of diazepam and its metabolites excreted: 20%. Temazepam (6.6%), desmethyldiazepam (3.9%) and oxazepam (2.8%) (Chiba et al., 1995).	Metabolized in the liver to produce nordiazepam that is further converted in oxazepam e temazepam (Ariffin et al., 2007) After a single dose of diazepam, urinary concentrations of desmethyldiazepam, temazepam e oxazepam were 29.6 ± 22.3, 57.4 ± 47.0 and 18.4 ± 16.7 ng/mL (Chiba et al., 1995) 0.5 - 0.2%, 3.6 - 4.4%, 9.0 - 6.4%, and 8.7 - 6.3% of a single ingested dose were excreted into the urine as diazepam, desmethyldiazepam, temazepam and oxazepam, respectively (Chiba et al., 1995).
NORDIAZEPAM	
-	Metabolized to oxazepam and oxazepam glucuronides (Moffat, 2005).
TETRAZEPAM	
13 – 49% as diazepam (Pavlic et al., 2007).	Principal metabolites are 3-hydroxy-tetrazepam, norhydroxytetrazepam, diazepam and nordazepam (Pavlic et al., 2007).
LORAZEPAM	
Negligible amounts unchanged (Ghasemi et al., 2006).	Extensively metabolized to its glucuronide conjugate (Ghasemi et al., 2006).
OXAZEPAM	
75% unchanged (Carballa et al., 2008) 70 to 80% is excreted in the urine almost entirely as oxazepam glucuronide with traces of unchanged oxazepam	-

Excretion rates	Additional information
and other minor metabolites. 10% is eliminated in the feces unchanged (Moffat, 2005).	
ALPRAZOLAM	
About 80% of a dose is excreted in urine of which 11% is the unchanged drug, 15% α-hydroxyalprazolam and 9% benzophenone metabolite. Approximately 7% of a dose in excreted in feces (Moffat, 2005).	-
CARBAMAZEPINE	
10% unchanged; 25% as the dihydroxy metabolite and 2% as the 10,11 – epoxide (Moffat, 2005)	Main metabolites: 10,11-epoxycarbamazepine (as biologically active as the parent compound), hydroxylated and conjugated compounds (glucuronides) (Brunton, 2008)
CITALOPRAM	
12-20 % unchanged (Rao, 2007)	Metabolites: desmethylcitalopram, didesmethylcitalopram, citalopram-*N*-oxide and a propionic acid derivative. The metabolites have some pharmacological activity (Moffat, 2005).
FLUOXETINE	
<5% of the parent compound excreted (Jjemba, 2006)	Principal metabolites include fluvoxamine and norfluoxetine (Chouinard et al., 1999)
<10% unchanged parent compound in urine (Brooks et al., 2003a; Moffat, 2005)	Principal metabolite: norfluoxetine in urine (Brooks et al., 2003a; Moffat, 2005)
20-30% remains unchanged in urine (Fong and Molnar, 2008)	The metabolite norfluoxetine is biologically active and considered to be a more potent SSRIs when compared to its precursor (Fong and Molnar, 2008)
SERTRALINE	
Less than 0.2% are excreted unchanged in urine (Moffat, 2005)	It suffers extensive metabolization in the body through: N-demethylation, oxidative deamination and subsequent reduction, hydroxylation and glucuronide conjugation (Moffat, 2005)
NORSERTRALINE	
-	Norsertraline is a *N*-demethylated metabolite of sertraline. It is eliminated more slowly than sertraline and has pharmacological activity (Brunton, 2008)
PAROXETINE	
2% as parent compound (Moffat, 2005) in urine and <1% in feces (Cunningham et al., 2004).	-
62% and 36% excreted in urine and feces, respectively, in the form of inactive metabolites (Cunningham et al., 2004).	

Excretion rates	Additional information
VENLAFAXINE	
Excreted in urine: 1 to 10% as the unchanged drug, 30% *O*-desmethylvenlafaxine, 6 to 19% *N,O*-didesmethylvenlafaxine and 1% *N*-desmethylvenlafaxine. 2% is excreted in feces (Moffat, 2005).	-
AMITRIPTYLINE	
Negligible amounts unchanged (Kasprzyk-Hordern et al., 2008). 50% is excreted as 10–hydroxynortriptyline and its glucuronide conjugate and 27% as 10 - hydroxyamitriptyline; unchanged drug constitutes less than 5% of the excreted material. 8% may be eliminated in the feces as parent compound (Moffat, 2005).	Excreted as nortriptyline, 10-hydroxyamitriptyline (active), 10-hydroxynortriptyline (active) (Kasprzyk-Hordern et al., 2008).
PHENOBARBITONE	
6 – 39% of the parent compound excreted (Jjemba, 2006). 35% excreted unchanged (Kar, 2007). 25% is excreted in urine as unchanged drug and up to about 17% as 4–hydroxyphenobarbital (half of which in the form of glucuronide conjugate) (Moffat, 2005).	Enzymatic hydroxylation and carboxylation of aliphatic side chains following conjugation to glucuronides (Peschka et al., 2006). Major metabolites: *N*-glucopyranosylphenobarbital and 4–hydroxyphenobarbital and its glucuronide conjugate (Moffat, 2005).
MEPROBAMATE	
90% is excreted in urine. About 10 to 20% of the dose is excreted as unchanged drug and the remainder as metabolites (Moffat, 2005).	Major metabolites: 2–hydroxypropylmeprobamate and meprobamate *N*-glucuronide (Moffat, 2005).
PENTOBARBITONE	
1% remained unchanged in urine (Moffat, 2005).	-
BUTALBITAL	
About 5% is excreted in urine as the parent compound (Moffat, 2005).	Major metabolite excreted in urine: 5-(2,3–dihydroxypropyl)-5–isobutylbarbituric acid (20 to 60%) (Moffat, 2005).

2.4 RESISTANCE TO WWTPS REMOVAL METHODS AND OCCURRENCE IN SLUDGE

Generally, literature data on pharmaceuticals in the environment suggest that the large majority (if not all) of urban wastewaters are contaminated with these compounds (Gros et al., 2010; Jelic et al., 2011). An approximate prediction about the type and abundance of active substances which can be found in raw wastewaters can be done according to the local rates of consumption, which are a first indication of the pharmaceutical compounds that might be present in more significant amounts (Ternes, 2005). The study of the behavior of pharmaceuticals through WWTPs is a noteworthy tool to assess the probable concentrations of these compounds in the final treated effluents and sludge, allowing for the evaluation of the effects of discharging them into the environment (Jones et al., 2005b).

It is now well-established that pharmaceuticals are not efficiently removed by wastewater treatments, being present in significant amounts in WWTPs effluents and sludge (Ternes, 1998; Debska et al., 2004; Xia et al., 2005; Gómez et al., 2007; Conkle et al., 2008; Zhang et al., 2008; Loganathan et al., 2009; Jelic et al., 2011) (see tables 2.1 – 2.3, section 2.2, for data about pharmaceuticals in WWTPs effluents and sludge). Actually, WWTPs were not specifically intended to remove bioactive xenobiotics and removal efficiencies can range from zero to almost complete removal, depending on specific treatments used in more or less sophisticated WWTP facilities (Petrovic and Barceló, 2007). Biological degradation and sorption are the most common mechanisms applied in WWTP; though, as it will be shown below, the removal efficiencies are not always satisfactory and more advanced techniques should be applied. However, even with the present knowledge, municipal WWTPs have been hardly focused on the removal of trace organic pollutants, such as pharmaceuticals. Even in the case of more sophisticated WWTPs, the large diversity of trace organic compounds and the difficulty of predicting individual responses to more advanced treatments illustrate the complexity of this issue (Ternes, 2005; Jelic et al., 2011). It must also be emphasized that following the disappearance of a pharmaceutical in the liquid phase is not sufficient to conclude that it was completely removed as it may pass into the solid phase, or exist in a different form of the parent compound due to chemical transformations (Petrovic and Barceló, 2007).

Final treated effluents are commonly discharged in surface waters; this can lead to indirect reuse of wastewaters in areas where these surface waters are a source of potable water (Drewes et al., 2001; Jones et al., 2005a; Glassmeyer et al., 2008). This indirect water reuse (planned or unplanned, due to water overflows or leaks in the sewage systems) might lead to the presence of

pharmaceuticals in drinking water and yet, these compounds are not contemplated in the Drinking Water Directive 98/83/EC. Also, WWTPs effluents are increasingly being used for irrigation of crops and arid areas and groundwater recharge in several countries throughout the world (Drewes et al., 2001; Pedersen et al., 2005; Glassmeyer et al., 2008). Additionally, the use of Slow Rate Systems (SRS) to purify wastewater is becoming more and more common, consisting on its application on land, taking advantage of physical, chemical, and biological mechanisms (such as filtration, degradation, soil adsorption, chemical precipitation, denitrification, volatilization, and plant uptake) that occur concurrently in the soil-water-atmosphere environment (Paranychianakis et al., 2006). All these practices have the obvious advantage of reducing the demand for water supplies (which is a main concern due to the scarcity of potable water), but constitute viable pathways for the introduction of pharmaceuticals in soils, surface and ground waters (and ultimately in drinking waters) through runoff and infiltration (Pedersen et al., 2005).

Several psychiatric drugs partially or totally resist wastewater treatments. In Table 2.5 are gathered some data concerning the removal efficiency of the anxiolytic diazepam and of the anti-epileptic carbamazepine. In both cases, several methods (including the most commonly used) have removal efficiencies bellow 10%, being evident that a large amount of diazepam and carbamazepine passes unaffected through WWTPs. Only more advanced methods, such as advanced oxidation processes and ultra and nanofiltration are able to remove diazepam and carbamazepine almost entirely, (50-90 or more than 90% of efficiency). These facts justify the frequent occurrence of these pharmaceuticals in environmental samples. In Table 2.6 it is shown a set of data focused on fluoxetine, meprobamate, diazepam and carbamazepine. Again, in general, all these compounds are particularly resistant to more advanced treatment processes (less than 30% of removal); globally the best results were obtained with ozonation or ozonation/peroxidation which were already pointed out as good efficient alternatives (yet, very energy consuming) (Ternes, 2005). Note that, due to the inefficiency of the large majority of the advanced treatment processes presented here, meprobamate (a barbiturate) was recently identified in finished drinking waters in the United States (Snyder, 2008; Benotti et al., 2009).

In WWTPs it is very likely to occur a deconjugation phenomenon, as stated before (Jones et al., 2005b). The most relevant consequence of this deconjugation process is the increase of biologically active compounds in influents and, consequently, in effluents and sludges (Ternes, 1998; Petrovic and Barceló, 2007). The hypothesis on the deconjugation of pharmaceutical conjugates is supported by investigations which concluded that the concentration of parent compounds, found in WWTPs effluents, is considerable higher than the concentration of the conjugated form, contradicting the excretion patterns and underlining the possibility of, at least,

partial cleavage of the conjugates (Ternes, 1998). Gros et al. (2010) reported that carbamazepine, several benzodiazepines and SSRIs suffered no elimination through the selected WWTPs, presenting higher concentration in effluents than in influents, corroborating this hypothesis.

Table 2.5. Removal of diazepam and carbamazepine in wastewater treatment plants; adapted from the EU-Project POSEIDON final report, 2005 (Ternes, 2005).

Removal treatment method	% Removal	
	Diazepam	Carbamazepine
Primary treatment	< 10	< 10
COD removal (SRT* ≤ 2 days)	< 10	< 10
Nitrification (SRT 10 to 15 days)	< 10	< 10
Sludge stabilization (SRT ≥ 25 days)	< 10	< 10
Membrane bioreactor (SRT ≥ 25 days)	No data	< 10
Biofilter	No data	< 10
Soil, unsaturated zone	No data	< 10
Groundwater, saturated zone	10 - 50	< 10
Sludge anaerobic treatment	10 – 50	10 - 50
Fenton process	< 10	< 10
Effluent ozonation	10 - 50	> 90
Ozonation	10 - 50	> 90
AOPs (Advanced oxidation processes)	50 -90	50 -90
GAC (Granular activated carbon)	> 90	> 90
Ultrafiltration/PAC(Powdered activated carbon)	> 90	> 90
Nanofiltration	> 90	> 90
Chlorination	< 10	< 10
Chlordioxide	< 10	< 10

*SRT: Sludge retention time

Table 2.6. Removal percentages of diazepam, fluoxetine, carbamazepine and meprobamate by several methods applied in WWTPs.

Treatment method	< 30%	30 – 70%	> 70%	References
Free Chlorine (3.5 mg L^{-1})	Diazepam Fluoxetine Meprobamate Carbamazepine			(Snyder, 2008)
UV at 40 mJ cm^{-2}	Diazepam Fluoxetine Meprobamate Carbamazepine			(Snyder, 2008)
Ozone (2.5 mg L^{-1} dose)		Meprobamate	Diazepam Fluoxetine Carbamazepine	(Snyder, 2008)
Powder activated carbon (PAC)	Meprobamate (0%)	Diazepam (53%) Carbamazepine (55%)	Fluoxetine (92-96%)	(Westerhoff et al., 2005) (Shon et al., 2006)
Flocculation with ferric chloride	Diazepam (0%) Fluoxetine (0%) Meprobamate (0%) Carbamazepine (0%)			(Shon et al., 2006)
Flocculation with Aluminum	Diazepam (5%) Fluoxetine (20%) Meprobamate (0%) Carbamazepine (7%)			(Shon et al., 2006)
Chlorination pH 5.5	Fluoxetine (20%) Meprobamate (16%)		Diazepam (71%) Carbamazepine (98%)	(Shon et al., 2006)
Ozone/H_2O_2		Meprobamate (61%)	Diazepam (85%); Fluoxetine (98%) Carbamazepine (98%)	(Shon et al., 2006)
Ultrafiltration	Diazepam (7%) Fluoxetine (0%) Meprobamate (0%) Carbamazepine (0%)			(Shon et al., 2006)
Nanofiltration		Meprobamate (32%) Carbamazepine (61%)	Diazepam (75%) Fluoxetine (92%)	(Shon et al., 2006)

It must also be considered the presence of psychiatric pharmaceuticals in sludge which result, inevitably, from wastewater treatments (Chenxi et al., 2008; Lapen et al., 2008; Gielen et al., 2009). In the United States, a WWTP produces an average of 240 kg (dry weight) of biosolids per million liters of treated wastewater (Kinney et al., 2006); in the European Union, it is estimated to be produced between 9 and 38 kg (dry weight) of biosolids per capita per year (Lapen et al., 2008). Knowledge about sorption of pharmaceuticals onto the solid phase is crucial to determine removal rates, bioavailability, transport between different environmental compartments and the rate of physico-chemical transformations (Caliman and Gavrilescu, 2009). Benzodiazepines and carbamazepine are characterized by moderate lipophilicity (Chouinard et al., 1999), low-medium octanol-water partitioning coefficients (e.g. 2.19 for temazepam, 2.7 for diazepam (Stein et al., 2008) and 2.45 for carbamazepine (Hanna et al., 1998)) and relatively low water solubility; thus, these compounds might partition moderately into the solid phase. However, the sorption behavior might be mediated by different types of interactions depending also on the organic content of the solid phase and it is, overall, difficult to predict the relevance of sorption processes for each case (Loffler et al., 2005; Stein et al., 2008; Yamamoto et al., 2009; Yu et al., 2009). A recent study by Jelic et al. (2011) indicated that sorption onto sludge was responsible for the removal of approximately 20% of the initial concentration of diazepam in the influent of 3 WWTPs. This indicates that, in this case, while sorption onto sludge contributes to the removal of this compound from the liquid effluent, it is not the main mechanism of elimination from aquatic compartments, being consistent with a low-medium sorption capacity. Relatively to antidepressants, Kinney et al. (2006) found fluoxetine in 9 samples of biosolids collected in 8 different WWTPs with considerably high concentrations in the range of 100 to 4700 μg kg^{-1} (in the same study carbamazepine was also found in biosolids with concentrations between 15 and 1220 μg kg^{-1}). These relatively high sorptions to biosolids enable the removal of the pharmaceuticals from wastewaters. However, the biosolids produced in WWTPs are used in agricultural soils, large-scale landscaping, domestic landscaping and soil-surface revegetation (Jones et al., 2005b; Kinney et al., 2006; Lapen et al., 2008) and constitute a vehicle of these compounds into the environment. More detailed data about the sorption and persistence of psychiatric drugs in soils/sediments will be given along the next sub-chapter.

2.5 (A)BIOTIC DEGRADABILITY AND PERSISTENCE IN THE ENVIRONMENT

The degradability (through abiotic or biotic processes) and persistence of pharmaceuticals in water/sediment compartments are of great importance to the assessment of chronic exposure of organisms living in these environments. However, despite the strong evidences of considerable

high persistence and resistance to (bio)degradation of compounds from this pharmaceutical group, few data is available on this issue. The available literature is essentially focused on carbamazepine, the most studied psychiatric pharmaceutical (Andreozzi et al., 2003; Doll and Frimmel, 2003; Lam and Mabury, 2005; Chiron et al., 2006; Matamoros et al., 2009). Few studies are related to some antidepressants and anticonvulsants (Kwon and Armbrust, 2005; Kwon and Armbrust, 2006; Peschka et al., 2006) and research concerning benzodiazepines is, up to date, very scarce, especially photodegradation and biodegradation studies.

2.5.1 Anxiolytics, sedatives and hypnotics

Generalized occurrence of diazepam in rivers, lakes and WWTP influents and effluents, suggests limited degradation of these compounds under environmental conditions (Redshaw et al., 2008). A study on the biodegradability of diazepam and related pharmaceuticals (oxazepam and temazepam), performed in liquid and solid matrices containing bacterial cultures typical of sewage sludge-amended soils, reported that diazepam is the most persistent of the considered pharmaceuticals. No losses caused by biotic or abiotic factors were observed during a 60 days experiment. Oxazepam underwent a loss of 40% due to biodegradation but the study also revealed the hypothesis of being transformed in another biologically active and persistent metabolite (Redshaw et al., 2008). Loffler et al. (2005) also considered diazepam as a highly persistent pharmaceutical, with a dissipation time (DT_{90}) >> 365 days and oxazepam as moderately persistent in water/sediment systems. According to this study, diazepam suffers rapid and extensive sorption onto sediments; it is highly stable in soils and during wastewater treatments (Loffler et al., 2005) and remains stable in ground waters (Ternes, 2005). Photodegradation of diazepam is also considered to constitute a feasible mechanism for the decrease of its concentration in surface waters (Boreen et al., 2003; Kummerer, 2008). However, there are no studies corroborating the relevance of this hypothesis under environmental conditions. Overall, literature data suggest that diazepam is potentially being accumulated in the environment. Note that differences in behavior between several benzodiazepines, which are detailed in some of the referred investigations, are associated with small differences in functional substituent groups. This fact underlines the importance of analyzing a wider range of extensively prescribed benzodiazepines to minimize the lack of information on the fate and persistence of this type of pharmaceuticals. Nevertheless, up to date, there are no references to studies concerning other environmentally occurring benzodiazepines, such as alprazolam and lorazepam.

Another relevant study related with the persistence of anxiolytics, sedatives and hypnotics is presented by Peschka et al. (2006). The biotic and abiotic degradability of several barbiturates (butalbital, secobarbital, hexobarbital, aprobarbital, phenobarbital, and pentobarbital) were assessed and barbiturates were subjected to biodegradability under aerobic conditions and hydrolysis. None of the barbiturates showed any evidence of degradation, stressing their high stability in the environment (Peschka et al., 2006). This behavior was also suggested by two distinct investigations that reported the identification of barbituratures in landfills which did not receive industrial or domestic wastes for more than 20 years (Eckel, 1993; Holm et al., 1995; Jones et al., 2001).

2.5.2 Anti-epileptics - Carbamazepine

When compared to benzodiazepines, much more is known concerning the environmental persistence and fate of the anti-epileptic carbamazepine. In the last few years, this drug has been a preferential target of the scientific community, mainly due to its generalized occurrence.

Several studies reported that carbamazepine is considerably more resistant to photodegradation than several other pharmaceuticals, which is crucial for the determination of its environmental half-life time in aquatic systems exposed to sunlight irradiation (Pal et al., 2010). Andreozzi et al. (2003) demonstrated that carbamazepine exposed to sunlight (in different seasons and at different latitudes) can persist in aquatic environments for more than 100 days. Under simulated irradiation conditions, several studies indicated that carbamazepine has half-life times in the order of 100 h (Andreozzi et al., 2002; Lam and Mabury, 2005; Yamamoto et al., 2009). Doll and Frimmel (2003) also showed that it is more resistant to photodegradation than the other tested compounds (clofibric acid and iomeprol), but environmental half-life times are not evidently presented. While it is clear that the literature suggests limited degradation of carbamazepine under solar irradiation, it is also difficult to predict real environmental half-life times and to compare different studies performed at different simulated irradiation conditions. Most importantly, the relevance of water composition in the persistence of contaminants should not be forgotten, due to the contribution of indirect photodegradation processes that might be triggered by, for instance, naturally occurring dissolved organic matter, nitrates and chloride. The influence of water components in the elimination of carbamazepine from the environment is not trivial and several investigations described that they can exert contrary effects; for example, Lam and Mabury (2005) and Matamoros et al. (2009) reported that dissolved organic matter was responsible for an enhancement of carbamazepine photodegradation rates while Chiron et al. (2006) reported that dissolved organic matter caused a decrease in the photodegradation. Moreover, some of the

photodegradation products already identified in literature are known to produce more adverse effects than carbamazepine itself. This is the case of acridine, one of the most frequently identified photoproducts, that is considered a mutagenic and carcinogenic compound (Chiron et al., 2006; Kosjek et al., 2009). Some literature studies also concluded that biodegradation processes hardly contribute to the decrease of carbamazepine concentrations (Stamatelatou et al., 2003; Joss et al., 2006; Zhang et al., 2008; Yamamoto et al., 2009; Pal et al., 2010). Concerning its fate in the water/soil(sediment) interface, and similarly to benzodiazepines, carbamazepine also presents low to medium sorption capacities and results may differ significantly according to the soil/sediment composition (Scheytt et al., 2005; Chefetz et al., 2008). Overall, it is considered that a significant amount of carbamazepine will remain in the aqueous phase (Zhang et al., 2008). As a consequence, and also due to its high resistance to bio and photodegradation, carbamazepine has been repeatedly proposed as an adequate marker of anthropogenic pollution (Clara et al., 2004; Glassmeyer et al., 2008; Yu et al., 2009). Thus, the assessment of carbamazepine contamination levels might constitute a valuable tool for the identification of relevant focal points of anthropogenic pollution.

2.5.3 Antidepressants

Published information on the (bio)degradability of SSRIs stated that these pharmaceuticals are potentially persistent, remaining in aquatic environments for relatively long periods of time and raising several concerns about environmental accumulation (Kwon and Armbrust, 2006; Paterson and Metcalfe, 2008; Redshaw et al., 2008; Kosjek and Heath, 2010). Several experiments have already demonstrated that fluoxetine and norfluoxetine are resistant to biodegradation processes occurring in liquid and soil cultures of bacteria of sewage sludge-amended soils (Kwon and Armbrust, 2006; Redshaw et al., 2008). No fluoxetine losses were observed during a 270 days assay (Redshaw et al., 2008). Kwon and Armbrust (2006) also concluded that fluoxetine is hydrolytically and photolytically stable in aquatic environments (with half-lives greater than 100 days). However, a distinct study reported that, under simulated conditions, fluoxetine has half-life times of 55 h and 5.5 – 22 h in deionized water and synthetic field water, respectively (Lam et al., 2005). The photo resistance of citalopram was also studied, indicating environmental half-life times of 14 to 65 days, depending on aqueous matrix composition (Kwon and Armbrust, 2005). Again, as stressed out in the previous sub-chapter, half-life times are dependent on different experimental conditions (natural or simulated sunlight – and, in this case, adopted lamp and irradiance levels); most of the times it is unclear how did the authors convert half-life times under simulated conditions to sunlight days. Therefore, it is difficult to predict real environmental half-life times and to properly compare different studies, and a uniform normalization of the results is lacking.

According to some literature, the major concern about fluoxetine environmental fate should not be focused on its persistence in water but in the sediments/soils distribution. Fluoxetine rapidly dissipates from water compartments as a result of high adsorption to sediments and soils, where it appears to be greatly persistent (Kwon and Armbrust, 2006; Redshaw et al., 2008). In general, SSRIs have sorption capacities greater than 91% (Kwon and Armbrust, 2008) and yet, these values could not be explained by high octanol-water partition coefficient (K_{ow}) (actually, benzodiazepines have higher K_{ow} and lower sorption capacities). The K_{ow} is related with the compounds hydrophobicity (Sabljic et al., 1995). For neutral and hydrophobic pharmaceuticals it is known that the organic carbon content of biomass, soils or sediments may be related with sorption mechanisms and therefore plausibly well correlated with K_{ow} (Kwon and Armbrust, 2008). In the particular case of SSRIs, log K_{ow} values vary from 1.12 (fluvoxamine) to 1.39 (citalopram); additionally, these pharmaceuticals also have relatively high water solubilities (from 3.022 to 15.460 mg L^{-1}). Hence, hydrophobic interactions could not justify the sorption mechanism of these pharmaceuticals and, consequently, K_{ow} is not an adequate parameter to properly predict their fates in environmental conditions (Kwon and Armbrust, 2008). A study performed by Wells (2006) presented D_{ow}, the pH dependent octanol-water distribution ratio (a combination of K_{ow} and pK_a) as a more appropriate physicochemical parameter to understand the distribution of pharmaceuticals in water/sediment systems. This parameter takes into account the hydrophilic character of a pharmaceutical at a specific pH, allowing to understand the mobility of a compound at environmentally relevant pH conditions. Also, multiple mechanisms may be useful to describe the unexpectedly high SSRIs sorption onto soils/sediments based, for instance, on cation exchange, cation bridging at clay surfaces, surface complexation or hydrogen bonding (Kwon and Armbrust, 2008).

As far as other antidepressants are concerned, very limited data is available, resulting in a substantial lack of knowledge about these emerging environmental pollutants. However, the information gathered indicates that persistence and accumulation are very likely to occur. One example is the case of the tricyclic antidepressant amitriptyline which was shown to be non biodegradable under sewage treatment conditions (Halling-Sorensen et al., 1998), highlighting the importance of performing more studies on the (bio)degradation of these pharmaceuticals in environmentally relevant conditions and to find newer solutions to promote their elimination.

2.6 TOXICITY OF PSYCHIATRIC DRUGS FOR NON-TARGET ORGANISMS

Psychiatric pharmaceuticals occur in the environment in the range of ng L^{-1} to μg L^{-1}. Although these concentrations are below the predicted levels to cause harm to humans, as well as to cause acute toxicity to non-target organisms, it is pertinent to take into account that these compounds do not occur isolated but as complex mixtures, such that additive and synergistic effects might occur (Bound et al., 2006; Pomati et al., 2008; Quinn et al., 2009; Schnell et al., 2009; Styrishave et al., 2011). In this context, and due to having an intrinsic biological activity that can affect nervous and endocrine systems, psychiatric pharmaceuticals are one of the most significant groups in what concerns the evaluation of ecotoxicological effects in terrestrial and aquatic non-target organisms (van der Ven et al., 2006). Furthermore, the increasing number of studies about chronic toxicity on non-target aquatic organisms point out that no extrapolations between acute and chronic toxicity should be done, underlining the need to develop a distinct approach to better clarify this issue (Cunningham, 2006; Kummerer, 2008). In fact, seeing that aquatic organisms are extensively exposed to pharmaceuticals, it would be more important to understand life cycle toxicity rather than perform acute toxicity tests (Halling-Sorensen et al., 1998; Petrovic and Barceló, 2007).

2.6.1 Anxiolytics, sedatives and hypnotics

The toxicity data for diazepam are gathered in Table 2.7. Actually, to the best of our knowledge, diazepam is the only benzodiazepine with anxiolytic, sedative or hypnotic properties whose toxic effects were studied and evaluated. Interestingly, only one study was found concerning effects due to other anxiolytics (buspirone and chlordiazepoxide) (Bencan et al., 2009). Acute toxicity tests on several aquatic organisms revealed that the concentrations needed to the observation of acute adverse effects caused by diazepam (in the mg L^{-1} scale) are well above the environmental concentrations of diazepam – the maximum concentration detected in surface waters was 0.88 μg L^{-1} (Ternes, 2001). Nonetheless, a study performed by Pascoe et al. (2003), comparing acute and chronic toxicity to an aquatic invertebrate sedentary organism (*Hydra vulgaris*), described visible adverse effects (deficient regeneration of polyps) at concentrations of 10.0 μg L^{-1}. More recently, Oggier et al. (2010) showed that diazepam affects gene expression in zebrafish, after 14 days of exposure, at levels as low as 235 ng L^{-1}. This concentration is lower than the concentration found in some surface waters and effluents of WWTPs. This is a clear first indication that diazepam is able to exert effects on non-target organisms at environmentally relevant

concentrations, after a chronic exposure. With these findings, and as the continuous discharge of these compounds in the environment results in exposures during the entire life cycle of the organisms (Halling-Sorensen et al., 1998; Pascoe et al., 2003), it is of great relevance to refer the inexistence of more chronic toxicity studies for other organisms or for other pharmaceuticals with the same mode of action.

2.6.2 Anti-epileptics - Carbamazepine

In the last few years there has been a significant increase in the number of studies dealing with the ecotoxicity of carbamazepine. Some relevant data are gathered in Table 2.8.

Similarly to benzodiazepines, the majority of literature data reports that acute toxic effects caused by exposure to carbamazepine are not likely to occur at concentration levels found in the environment (Ferrari et al., 2003; Jos et al., 2003; van den Brandhof and Montforts, 2010; Li et al., 2011). Nevertheless, there is one literature study describing acute toxic effects (48 h of exposure) related to changes in the oxidative metabolism of cnidarians at concentrations that might cause some concern (7 - 47 μg L^{-1}) (Quinn et al., 2004); note that the maximum concentration of carbamazepine found until now, in surface waters, was approximately 11.6 μg L^{-1} (Loos et al., 2009). Another relevant investigation also found that carbamazepine might be able to induce changes in the reproduction of *C. dubia* at concentrations that are reasonably close to the environmental concentrations (no observable effects concentration, NOEC, 25 μg L^{-1}; lowest observable effects concentration, LOEC, 100 μg L^{-1}), during a 7 day exposure (Ferrari et al., 2003). In addition, Quinn et al. (2009) showed that complex mixtures of pharmaceuticals can exert the same toxic effect in concentrations that are 2 to 3 orders of magnitude lower than the relevant pharmaceutical alone. The pharmaceutical mixture studied comprised carbamazepine and concentrations causing sub-lethal effects in a freshwater cnidarian were found to be in the range between ng L^{-1} and μg L^{-1}. These studies evidence that concentrations of carbamazepine found in the environment might not be as innocuous as it has been concluded in some investigations (focused in individual acute toxicity data).

Table 2.7. Toxicity data of diazepam for non-target organisms.

Test species	Acute toxicity Test	Acute toxicity data	Chronic toxicity data	Additional information	Reference
			DIAZEPAM		
Daphnia magna (Invertebrate)	EC_{50}, 24h	14.1 mg L^{-1}	-	-	(Calleja et al., 1994)
	EC_{50}	13.9 mg L^{-1}; 4.3 mg L^{-1}	-	-	(Halling-Sorensen et al., 1998)
	LC_{50}	13.9 mg L^{-1}	-	-	(Lilius et al., 1995)
	LC_{50}	4.3 mg L^{-1}	-	-	(Halling-Sorensen et al., 1998)
Hydra vulgaris (Invertebrate, cnidarian)	-	<1 mg L^{-1}	10.0 µg L^{-1}	Evaluation of the capacity for regenerate polyps	(Pascoe et al., 2003)
Artemia parthenogenetica (crustacean)	LC_{50}	12.2 mg L^{-1}	-	The most toxic compound between clofibric acid, SDS and clofibrate	(Nunes et al., 2005)
Tetraselmis chuii (unicellular marine algae)	IC_{50}	16.5 mg L^{-1}	-	The most toxic compound between clofibric acid, SDS and clofibrate	(Nunes et al., 2005)
PLHC-1 and RTG-2 cell lines (Fish)	EC_{50}	0.363 - 0.604 mM (103 - 172 mg L^{-1})	-	-	(Caminada et al., 2006)
Gambusia holbrooki (euryhaline fish)	LC_{50}	12.7 mg L^{-1}	-	The second most toxic compound between clofibric acid, SDS and clofibrate	(Nunes et al., 2005)
Danio rerio (zebrafish)	-	-	-	Signs of significant anxiolytic effects after a 3 min exposure to 0 – 20 mg L^{-1}. The same was observed for the anxiolytic buspirone.	(Bencan et al., 2009)
	-	-	235 ng L^{-1}	Diazepam affected after 14 days of exposure both at 235 ng L^{-1} and 291 µg L^{-1} concentration levels; significant changes in locomotor activity in embryos at 291 µg L^{-1}	(Oggier et al., 2010)

Table 2.8. Toxicity data of the anti-epileptic pharmaceutical carbamazepine for non-target organisms.

Test species	Acute toxicity Test	Acute toxicity data	Chronic toxicity data	Additional information	Reference
			CARBAMAZEPINE		
Hydra attenuata (cnidarian)	Monitorization of oxidative metabolism, 48 h	0.03 – 0.2 µM (7 – 47 µg L^{-1})	-	Signs of lethal toxicity at 0.6 and 6 mM levels (142 and 1418 mg L^{-1}), after 48 h.	(Quinn et al., 2009)
Daphnia magna (Invertebrate)	EC_{50}, 48h	>13 800 µg L^{-1}	-	-	(Ferrari et al., 2003)
	EC_{50}, 24 and 48h	475 and 414 µM (112 and 98 mg L^{-1})	-	Indicator for EC_{50} test: immobilization, after 24 and 48 h, respectively.	(Jos et al., 2003)
Ceriodaphnia dubia (Invertebrates)	EC_{50}, 48h	77 700 µg L^{-1}	NOEC 25 µg L^{-1}; LOEC 100 µg L^{-1}	7 days chronic exposure	(Ferrari et al., 2003)
Pseudokirchneriella subcapitata (Algae - green)	-	-	-	Bioaccumulation factor of 2.2 after 24h exposure to 150 mg L^{-1}	(Vernouillet et al., 2010)
Vibrio fischeri (bacteria)	EC_{50}, 5, 15 and 60 min	370, 332 and 272 µM (87, 78 and 64 mg L^{-1})	-	Indicator for EC_{50} test: bioluminescence. Results after 5, 15 and 60 min, respectively	(Jos et al., 2003)
Brachionus calyciflorus (rotoxkit)	-	-	NOEC 377 µg L^{-1}; LOEC 754 µg L^{-1}	48h test used to calculate NOEC and LOEC	(Ferrari et al., 2003)
Oncorhynchus mykiss (rainbow trout)	LC_{50}, 96h	19.9 mg L^{-1}	-	-	(Li et al., 2011)
Danio rerio (fish)	EC_{50}, 72h	86.5 mg L^{-1}		NOEC – 30.6 mg L^{-1}	(van den Brandhof and Montforts, 2010)
Cyprinus carpio (common carp)	-	-	-	Evidences of oxidative stress in fish spermatozoa after 2h; range of tested concentrations: 0.2 – 20 mg L^{-1}	(Li et al., 2010)
Oryzias latipes (fish)	-	-	9 days of chronic exposure	Changes in feeding behavior and swimming speed after exposure to 6.15 mg L^{-1}	(Nassef et al., 2010)

2.6.3 Antidepressants

A large number of researches are focused on the potential environmental adverse effects of steroids and other estrogens, since it was discovered their interference in endocrine responses of aquatic organisms (Brooks et al., 2003a). On the contrary, little attention has been paid to non-steroidal pharmaceuticals that present the same ability, which is directly related to the action mode of antidepressants: not only can they affect the neuronal system but also disrupt neuro-endocrine signaling causing perturbations on the reproductive behavior (Foran et al., 2004; van der Ven et al., 2006; Henry and Black, 2008; Péry et al., 2008; Gust et al., 2009; Sánchez-Argüello et al., 2009). Detailed information on the toxicity of antidepressants to aquatic organisms can be found in Table 2.9. Amongst antidepressant pharmaceuticals, the SSRI fluoxetine has been the most extensively studied compound. Yet, the effects of this compound (and other antidepressants, in general) on aquatic organisms are not completely understood (Cunningham, 2006). A first interesting conclusion on the ecotoxicity of fluoxetine arises from the comparison between its acute toxicity data (Table 2.9) and acute toxicity data for diazepam or carbamazepine (Tables 2.7 and 2.8, respectively). While the majority of studies concerning diazepam or carbamazepine pointed out acute toxicity levels higher than 10 mg L^{-1}, fluoxetine acute toxic concentrations are, usually, well below 1 mg L^{-1} and several studies even reported acute toxic effects at concentrations as low as 20 – 40 μg L^{-1} (Brooks et al., 2003a; Brooks et al., 2003b; Johnson et al., 2007).

Fluoxetine, paroxetine and sertraline are possible examples of antidepressants that are suspected to be hormonally active (Kolpin et al., 2002). In primary producers, invertebrates and fish, the mechanistic responses to SSRIs are not completely clarified. However, several fish species were identified for the possession of serotonin receptors, making it possible to predict that SSRIs can modulate serotonin levels in these animals (Brooks et al., 2005). An investigation of chronic and acute toxicity of SSRIs to *Ceriodaphnia dubia*, by Henry et al. (2004), confirmed that the reproduction patterns of these invertebrates are affected by the exposure to these antidepressants. SSRIs were shown to have the ability of reducing the number of neonates and broods per female with a LOEC of 0.045 mg L^{-1}. This study also established that increasing concentrations of citalopram, fluvoxamine, fluoxetine, sertraline and paroxetine caused an increase in the mortality of *C. dubia*: LC_{50} values ranged from 0.12 mg L^{-1} for sertraline to 3.90 mg L^{-1} for citalopram. The locomotion capabilities of *C. dubia* were also negatively affected at SSRIs concentrations below the determined LC_{50} values (Henry et al., 2004).

Several other studies have already established a direct connection between exposure to SSRIs and gonadal maturation, induction of parturition, metamorphosis and spawning in aquatic

organisms (Henry et al., 2004; Fong and Molnar, 2008; Gust et al., 2009; Bringolf et al., 2010). Concentrations as low as 32 ng L^{-1} were shown to induce spawning in male zebra mussels (*Dreissena polymorpha*) (Fong, 1998). There are also experimental evidences that norfluoxetine induces spawning in zebra mussels and dark false mussels at concentrations of 1 μM, approximately 0.3 mg L^{-1} (Fong, 1998; Fong and Molnar, 2008). A similar study with fluoxetine showed that it also induces spawning in zebra mussels and dark false mussels but at concentrations between 10 to 20 times lower than norfluoxetine. Moreover, fluoxetine causes parturition in fingernail clams (*Sphaerium striatinum*) at concentrations of 10 μM (3 mg L^{-1}) (Fong, 1998; Fong et al., 1998; Fong and Molnar, 2008). Consequently, this class of pharmaceuticals may constitute a real concern as, in this particular case, the induction of spawning and parturition in the wrong time of the year may increase the percentage of early stage mortality due to the scarcity of food for juvenile development, resulting in serious negative consequences for these species (Fong and Molnar, 2008).

Of particular relevance are two recent researches that performed chronic toxicity tests and concluded that fluoxetine can cause changes in the behavioral response of *E. marinus* (crustacea) when present at concentrations of 10 ng L^{-1} (Guler and Ford, 2010). Also, fluoxetine can affect growth during metamorphosis stages of *X. laevis* (Tadpoles) at 10 μg L^{-1} (Conners et al., 2009). These concentrations are extremely low and commonly found in aquatic environments, especially those close to WWTP effluents discharge points. The validity of these studies implies the assumption that non-target aquatic organisms are actually being directly affected by the presence of fluoxetine.

Relatively to the possibility of bioaccumulation, the uptake and depuration of fluoxetine was investigated in freshwater fish species. Paterson and Metcalfe (2008) exposed the Japanese Medaka (*Oryzias latipes*) to a fluoxetine concentration of 0.64 μg L^{-1} during seven days followed by a 21 days period of depuration. The concentrations of fluoxetine and its active metabolite norfluoxetine increased during the uptake period and decreased during the depuration phase. Uptake of fluoxetine and norfluoxetine was observed in the first five hours of exposure, with a maximum concentration determined on the third day. The depuration phase permitted to determine the fluoxetine half-life in the fish tissues (9.4 ± 1.1 days), clearly indicating that fluoxetine and norfluoxetine can persist in biological tissues and present a large potential for bioaccumulation in fish.

Table 2.9. Toxicity data of antidepressants for non-target organisms.

Test species	Acute toxicity test	Acute toxicity data	Chronic toxicity data	Additional information	Reference
			FLUOXETINE		
Daphnia magna (Invertebrate)	EC_{50}	820 μg L^{-1}	-	-	(Brooks et al., 2003a)
	LC_{50}	820 μg L^{-1}	-	-	(Brooks et al., 2003b)
	-	-	429; 430 and 444 μg L^{-1} (LOEC survival test with R-fluoxetine, rac-fluoxetine and S-fluoxetine, respectively)	21 day chronic exposure	(Stanley et al., 2007)
	-	-	8.9 and 31 μg L^{-1} (NOEC and LOEC affecting the length of newborns, respectively)		(Péry et al., 2008)
Ceriodaphnia dubia (Invertebrate)	EC_{50}	234 μg L^{-1}	-	-	(Brooks et al., 2003a)
	LC_{50}	234 μg L^{-1}	-	-	(Brooks et al., 2003b)
	-	-	0.056 mg L^{-1}	-	(Brooks et al., 2003a)
	LC_{50}, 48h	0.51 ± 0.07 mg L^{-1} (n=3)	0.089 mg L^{-1} (NOEC affecting the mean number of neonates produced)	Range of acute test concentration: 0.19-2.92 mg L^{-1}	(Henry et al., 2004)
Xenopus laevis (Tadpoles)	EC_{50}	6.4 mg L^{-1}; 6.6 mg L^{-1}	-	-	(Richards and Cole, 2006)
	-	-	10 μg L^{-1} LOEC, reduced growth at metamorphosis	70 day chronic exposure through metamorphosis	(Conners et al., 2009)
	-	-	-	A delay in the development of tadpoles was observed, during a several week assay at 0.029 – 0.29 μg L^{-1}.	(Foster et al., 2010)
Hyalella azteca	EC_{50}	>43 μg kg^{-1}	-	-	(Brooks et al., 2003a)
Chironomus tentans	LC_{50}	15.2 mg kg^{-1}	-	-	(Brooks et al., 2003b)
	EC_{50}	15.2 μg kg^{-1}	-	-	(Brooks et al., 2003a)

Test species	Acute toxicity test	Acute toxicity data	Chronic toxicity data	Additional information	Reference
Echinogammarus marinus (Crustacea)	-	-	Changes in behavioral response and increased predation risk were observed at 10 ng L^{-1} with a peak at 100 ng L^{-1}	21 day chronic exposure. Range of tested concentrations: 10 ng L^{-1} – 10 µg L^{-1}	(Guler and Ford, 2010)
Potamopyrgus antipodarum (Mollusc gastropod)	-	-	13 and 69 µg L^{-1} (NOEC and LOEC affecting reproduction, respectively)		(Péry et al., 2008)
	-	-	33.3 and 100 µg L^{-1} (NOEC and LOEC affecting the cumulate number of neonates, respectively)	42 day chronic exposure	(Gust et al., 2009)
Pseudokirchneriella subcapitata	EC_{50}	24 µg L^{-1}	-	-	(Brooks et al., 2003a)
	IC_{10};IC_{50}, 96h	31.34 ± 1.93; 44.99 ± 1.76 µg L^{-1}	-	-	(Johnson et al., 2007)
Scendesmus acutus (Algae)	IC_{10};IC_{50}, 96h	55.60 ± 4.73; 91.23 ± 2.74 µg L^{-1}	-	-	(Johnson et al., 2007)
S. quadricauda (Algae)	IC_{10};IC_{50}, 96h	97.76 ± 13.54; 212.98 ± 16.13 µg L^{-1}	-	-	(Johnson et al., 2007)
Chlorella vulgaris (Algae)	IC_{10};IC_{50}, 96h	2901.57 ± 1218.97; 4339.25 ±446.09 µg L^{-1}	-	-	(Johnson et al., 2007)
Unspecified (Algae - green)	-	-	0.001 mg L^{-1}	-	(Crane et al., 2006)
Oryzias latipes (Japanese medaka)	LC_{50}, 96h	5.5, 1.3 and 0.20 mg L^{-1} at pH 7,8 and 9, respectively	-	-	(Nakamura et al., 2008)
PLHC-1 and RTG-2 cell lines (Fish)	EC_{50}	0.0110 - 0.0242 mM (3.4 – 7.5 mg L^{-1})	-	-	(Caminada et al., 2006)
Pimephales promelas (fish)	EC_{50}	705 µg L^{-1}	-	-	(Brooks et al., 2003a)
	LC_{50}	705 µg L^{-1}	-	-	(Brooks et al., 2003b)

Test species	Acute toxicity test	Acute toxicity data	Chronic toxicity data	Additional information	Reference
	LC_{50}, 48h	212; 198 and 216 μgL^{-1} for R-fluoxetine, rac-fluoxetine and S-fluoxetine, respectively	170; 174 and 101 $\mu g\ L^{-1}$ (LOEC survival test with R-fluoxetine, rac-fluoxetine and S-fluoxetine, respectively)	7 day chronic exposure	(Stanley et al., 2007)
Elliptio complanata, *Lampsilis cardium*, *Lampsilis fasciola*	-	300 – 3 000 $\mu g\ L^{-1}$, changes in reproductive behavior after 48 h – 96 h	-	-	(Bringolf et al., 2010)
			PAROXETINE		
Ceriodaphnia dubia	LC_{50}, 48h	0.58 ± 0.13 mg L^{-1} (n=3)	-	Range of acute test concentration: 0.22 - 6.96 mg L^{-1}	(Henry et al., 2004)
Xenopus laevis	EC_{50}	4.6 mg L^{-1}		-	(Richards and Cole, 2006)
			CITALOPRAM		
Ceriodaphnia dubia	LC_{50}, 48h	3.90 ± 0.27 mg L^{-1} (n=3)	4 mg L^{-1} (LOEC affecting the mean number of neonates produced)	Range of acute test concentration: 0.59 - 7.84 mg L^{-1}	(Henry et al., 2004)
Ceriodaphnia dubia	-	-	0.8 mg L^{-1}	-	(Crane et al., 2006)
			FLUVOXAMINE		
Ceriodaphnia dubia	LC_{50}, 48h	0.84 ± 0.41 mg L^{-1} (n=3)	0.366 mg L^{-1} (NOEC affecting the mean number of neonates produced)	Range of acute test concentration: 0.10 - 2.21 mg L^{-1}	(Henry et al., 2004)
Pseudokirchneriella subcapitata	IC_{10};IC_{50}, 96h	3987.38 ± 322.88; 4002.88 ± 42.52 $\mu g\ L^{-1}$	-	-	(Johnson et al., 2007)
Unspecified (Algae – green)	-	-	31 mg L^{-1}	-	(Crane et al., 2006)
Scendesmus acutus (Algae)	IC_{10};IC_{50}, 96h	2503.65 ± 328.78; 3620.24 ± 34.96 $\mu g\ L^{-1}$	-	-	(Johnson et al., 2007)
S. quadricauda (Algae)	IC_{10};IC_{50}, 96h	1662.91 ± 157.16; 3563.34 ± 118.94 μgL^{-1}	-	-	(Johnson et al., 2007)

Test species	Acute toxicity test	Acute toxicity data	Chronic toxicity data	Additional information	Reference
Chlorella vulgaris (Algae)	IC_{10};IC_{50}, 96h	6162.86 ± 814.30; 10208.47 ± 379.24 µg L^{-1}	-	-	(Johnson et al., 2007)
SERTRALINE					
Ceriodaphnia dubia	LC_{50}, 48h	0.12 ± 0.05 mg L^{-1} (n=3)	0.045 mg L^{-1} (LOEC affecting the mean number of neonates produced)	Range of acute test concentration: 0.04 - 1.84 mg L^{-1}	(Henry et al., 2004)
Pseudokirchneriella subcapitata	IC_{10};IC_{50}, 96h	4.57 ± 0.66; 12.10 ± 1.00 µg L^{-1}	-	-	(Johnson et al., 2007)
Scendesmus acutus (Algae)	IC_{10};IC_{50}, 96h	54.59 ± 6.52; 98.92 ± 6.74 µg L^{-1}	-	-	(Johnson et al., 2007)
S. quadricauda	IC_{10};IC_{50}, 96h	48.19 ± 3.27; 317.02 ± 21.46 µg L^{-1}	-	-	(Johnson et al., 2007)
Chlorella vulgaris	IC_{10};IC_{50}, 96h	152.73 ± 5.09; 763.66 ± 25.42 µg L^{-1}	-	-	(Johnson et al., 2007)
Xenopus laevis (Tadpoles)	EC_{50}	4.6 mg L^{-1}	-	-	(Richards and Cole, 2006)
	-	-	0.1 µg L^{-1} LOEC, reduced growth at metamorphosis	70 day chronic exposure through metamorphosis	(Conners et al., 2009)
RISPERIDONE					
Unspecified (Algae - cyanobacteria)	-	-	<100 mg L^{-1}	-	(Crane et al., 2006)
Unspecified (Algae - green)	-	-	<10.0 mg L^{-1}	-	(Crane et al., 2006)

2.7 ANALYTICAL METHODS FOR THE DETERMINATION OF PSYCHIATRIC PHARMACEUTICALS IN ENVIRONMENTAL MATRICES

One of the main challenges in monitoring the occurrence of psychiatric pharmaceuticals in waste, surface and ground waters (as for all pharmaceuticals, in general), is the lack of simple, sensitive and low cost analytical methods to quantify pharmacologically active substances (and their metabolites) in the concentration range of ng L^{-1} to μg L^{-1} (Ternes, 2001; Debska et al., 2004; Buchberger, 2007). However, in the last decade, major advances have been achieved with the development of new analytical methods that allow the quantification of trace amounts of pharmaceuticals (Debska et al., 2004; Kostopoulou and Nikolaou, 2008; Buchberger, 2011). The first efforts in the field of pharmaceutical trace analysis often resulted in single-analyte methodologies but, nowadays, the development of multi-analyte methodologies, capable of quantifying up to several dozens of pharmaceuticals is becoming more and more common (Gracia-Lor et al., 2011; Togola and Budzinski, 2008; Huerta-Fontela et al., 2010; López-Serna et al., 2010; Baker and Kasprzyk-Hordern, 2011). The development of robust analytical methodologies is of crucial importance taking into account that all steps involved in a proper environmental risk assessment would depend, inevitably, on the existence of reliable analytical data. One of the most important difficulties in analyzing pharmaceuticals in environmental matrices (such as wastewaters and sludge) relies on the complexity of the samples, being this another reason for the requirement of analytical techniques with very high resolution and extremely low quantification limits (Jones et al., 2005b; Kosjek and Heath, 2010; Buchberger, 2011). Hence, the fundamental step of this type of analysis is often considered to be sample preparation (Sliwka-Kaszynska et al., 2003; Ramirez et al., 2007).

In the particular case of psychiatric pharmaceuticals, some data concerning the performance of analytical methods optimized for their determination are presented in Table 2.10. Note that, despite the novelty of this issue, literature data about analytical methods for psychiatric pharmaceuticals rose exponentially in the last few years. Consequently, this section does not intend to present a comprehensive compilation and analysis of published papers but to give a general idea of state-of-the-art and progresses made.

The most frequently used techniques include GC and LC with mass spectrometry detection (Gros et al., 2006; Buchberger, 2007; Kot-Wasik et al., 2007; Terzic et al., 2008; Buchberger, 2011). Even so, LC related methods have been much more explored as GC methods are limited to be applied to classes of compounds which are easily volatized or, alternatively, which can be derivatized to volatile compounds without the emergence of undesirable side products.

LC-MS/MS is the most used technique due to its versatility, specificity and selectivity. One of the major disadvantages of LC-MS/MS methods is their susceptibility to matrix interferences, in special when they are associated with the electrospray ionization mode (ESI), which is undoubtedly the most used ionization technique in pharmaceutical trace analysis. These matrix interferences are related to the suppression of the analyte signals (most commonly) due to the co-elution of matrix components and leading to erroneous results (Kot-Wasik et al., 2007; Buchberger, 2011). Therefore, the use of this technique requires an extensive preliminary study of the matrix effects (Gros et al., 2006). To overcome this difficulty, Vasskog et al. (2006) developed a HPLC-(ESI)-MS method with a two-step sample cleaning procedure including SPE (solid-phase extraction) and LLE (liquid-liquid extraction), resulting in a very sensitive and selective method with limits of quantification in the order of pg L^{-1}. Another way of minimizing these effects was investigated by Chu and Metcalfe (2007) that optimized the HPLC-MS technique for the determination of antidepressants, using the atmospheric pressure chemical ionization (APCI) that is less affected by the mentioned matrix effects. Specifically, HPLC-(ESI)-MS was already developed for the quantification of diazepam in environmental water samples (Ternes, 2001; Debska et al., 2004; Ramirez et al., 2007), consisting on a sample pretreatment (filtration with glass filters, 1 μm pore diameter, at pH 7.0) and a SPE enrichment (column RP C18 using MeOH for the elution, evaporation to dryness in gentle stream nitrogen and redissolution in phosphate buffer) prior to HPLC-(ESI)-MS analysis. Recently, Schultz and Furlong (2008) developed a quantitative method for the determination of 8 antidepressants and 2 derivatives in environmental aqueous samples, comprising a pretreatment using SPE followed by LC-(ESI)-MS/MS. The analyzed samples were collected from several municipal wastewater effluents and from a waste-dominated stream. As stated before, attention is drawn into the development of multi-analyte methods and psychiatric pharmaceuticals have started to be commonly included in the selected group of analytes.

It is also possible to name some examples of GC/MS methods which were optimized for the determination of psychiatric pharmaceuticals in environmental samples (Brooks et al., 2005; Wille et al., 2005; Chu and Metcalfe, 2007; Kosjek and Heath, 2010). A large number of active substances included in this pharmacological group are considered as "neutral pharmaceuticals" – designation used for compounds which do not have acidic functional groups (Ternes, 2001). As a consequence, these pharmaceuticals can be enriched at neutral pH using SPE and sorbents can be subsequently analyzed by GC-MS without the need of derivatization (Ternes, 2001). Togola and Budzinski (2008) developed a GC-MS based method for the simultaneous determination of the anxiolytics and antidepressants amitryptiline, imipramine, doxepine, nordiazepam and diazepam without derivatization. This technique has also already been optimized for the determination of diazepam, consisting on an initial sample pretreatment (filtration with glass filters, 1 μm, at pH 7.5)

followed by an enrichment step using SPE (column RP C18 using MeOH for the elution). The sample was then analyzed by GC-MS using MSTFA for the derivatization (Ternes, 2001; Debska et al., 2004).

As can be concluded from the data presented in Table 2.10, SPE is the most commonly adopted pre-concentration procedure. Due to the moderate hydrofobicity of the majority of psychiatric pharmaceuticals, SPE reverse-phase materials are usually adequate. On the other hand, with the rising trend of developing multi-analyte methodologies, the majority of the studies here presented used new polymeric sorbents that allows satisfactory results when in the presence of a wide variety of hydrophobic/hydrophilic compounds (Madureira et al., 2009; González Alonso et al., 2010; López-Serna et al., 2010; Baker and Kasprzyk-Hordern, 2011; Buchberger, 2011).

Few examples of immunoanalysis applications and their comparison with reference analytical techniques were reported (Valentini et al., 2002; Debska et al., 2004; Huo et al., 2007; Shelver et al., 2008; Bahlmann et al., 2009). From these literature studies, only one presented the development of an immunochemical method for the determination of psychiatric pharmaceuticals in the environment (determination of carbamazepine in surface waters) (Bahlmann et al., 2009). Consequently, the potentialities of environmental immunoanalytical methods have not been properly explored, in particular, in the determination of psychiatric drugs. Immunochemical methods usually require only little sample preparation procedures and are characterized by high sensitivity. They are capable of analyzing large amounts of samples in just a few hours, being adequate for high-throughput analysis. Also, these methods usually involve fewer costs than the common chromatographic methods. For these reasons, immunochemical analysis may be an excellent tool to quick and inexpensive environmental screenings (Buchberger, 2007; Buchberger, 2011). Although immunoassay techniques are not suitable for the determination of several structurally different analytes, they will be certainly useful techniques in the determination of markers of environmental pollution, allowing to take conclusions on the contamination levels of selected areas. Diazepam, fluoxetine and carbamazepine were pointed out to be considered references relatively to other pharmaceuticals with similar mode of action and they might constitute good indicators of the presence of psychiatric drugs in environmental samples (Cunningham, 2006; Buchberger, 2007; Yu et al., 2009).

Table 2.10. Performance of several analytical methods optimized for the determination of psychiatric pharmaceuticals in environmental samples.

Method	Sample	Sample preparation	Recovery ± standard deviation	LOQ	References
			DIAZEPAM		
LC-MS/MS	Sludge	USE followed by SPE	37 ± 6% (activated sludge, absolute recovery); 59 ± 11% (activated sludge, relative recovery); 25 ± 3% (digested sludge, absolute recovery); 48 ± 10% (digested sludge, relative recovery)	20 ng g^{-1}	(Ternes et al., 1998)
LC-MS/MS	Surface waters and WWTP influents and effluents	SPE	106 ± 9% - 115 ± 9%	2.6 – 10 ng L^{-1}	(Gros et al., 2009)
LC-MS/MS	Surface waters close to effluent discharge	SPE	64.5 ± 9.8% - 75.1 ± 4.3%	2.60 ng L^{-1}	(Madureira et al., 2009)
UPLC – MS/MS	WWTP influents and effluents	SPE	88 ± 3%	1.5 ng L^{-1}	(Huerta-Fontela et al., 2010)
UPLC – MS/MS	Surface waters and WWTP influents and effluents	SPE	89 ± 2% - 105 ± 13% (SPE absolute recovery); 99 ± 3% - 103 ± 10% (SPE relative recovery)	0.5 – 6.0 ng L^{-1}	(Baker and Kasprzyk-Hordern, 2011)
GC-MS	Surface water and drinking water (n=5)	SPE	102 ± 14%	20 ng L^{-1}	(Ternes et al., 1998)
GC-MS	STP effluent (n=5)	SPE	102 ± 14%	100 ng L^{-1}	(Ternes et al., 1998)
GC-MS	Tap water	SPE	73%	22 ng L^{-1}	(Sacher et al., 2001)
GC-MS	Surface water	SPE	99%	22 ng L^{-1}	(Sacher et al., 2001)
			LORAZEPAM		
LC-MS/MS	Surface waters and WWTP influents and effluents	SPE	111 ± 11% - 138 ± 25%	6 – 24 ng L^{-1}	(Gros et al., 2009)
UHPLC-MS/MS	Surface waters and WWTP effluents	SPE	86 ± 2% - 96 ± 2% (surface water); 85 ± 2% - 87 ± 2% (WWTP effluents)	4.3 ng L^{-1} and 30 ng L^{-1}	(Gracia-Lor et al., 2010)

Method	Sample	Sample preparation	Recovery ± standard deviation	LOQ	References
UPLC – MS/MS	WWTP influents and effluents	SPE	101 ± 4%	1.5 ng L^{-1}	(Huerta-Fontela et al., 2010)
ALPRAZOLAM					
UHPLC-MS/MS	Surface waters and WWTP effluents	SPE	78 ± 4% - 83 ± 4% (surface water); 75 ± 4% - 78 ± 3% (WWTP effluents)	2.9 ng L^{-1} and 11 ng L^{-1}	(Gracia-Lor et al., 2010)
UPLC – MS/MS	WWTP influents and effluents	SPE	110 ± 5%	1.5 ng L^{-1}	(Huerta-Fontela et al., 2010)
OXAZEPAM					
UPLC – MS/MS	WWTP influents and effluents	SPE	75 ± 4%	0.02 ng L^{-1}	(Huerta-Fontela et al., 2010)
UPLC – MS/MS	Surface waters and WWTP influents and effluents	SPE	51 ± 3% - 62 ± 2% (SPE absolute recovery); 96 ± 4% - 205 ± 5% (SPE relative recovery)	0.5 – 5.9 ng L^{-1}	(Baker and Kasprzyk-Hordern, 2011)
BARBITURATES (BUTALBITAL, SECOBARBITAL, PENTOBARBITAL, HEXOBARBITAL, APROBARBITAL, PHENOBARBITAL)					
GC-MS	Rhine river water	SPE	64 ± 1% - 105 ± 1%	1 - 5 ng L^{-1}	(Peschka et al., 2006)
CARBAMAZEPINE					
LC-MS/MS	Surface water	SPE	93 ± 1% (high spiking level); 67 ± 6% (low spiking level)	10 ng L^{-1}	(Gros et al., 2006)
LC-MS/MS	WWTP effluents	SPE	97 ± 5% (high spiking level); 93 ± 12% (low spiking level)	40 ng L^{-1}	(Gros et al., 2006)
LC-MS/MS	Surface water	SPE	98 ± 3% (high spiking level); 105 ± 10% (low spiking level)	61 ng L^{-1}	(Gros et al., 2006)
LC-MS/MS	Surface waters	SPE	82.2 ± 8.1% (spike level: 50 ng L^{-1}) 73.5 ± 11.5% (spike level: 500 ng L^{-1})	48 pg L^{-1}	(Zhang and Zhou, 2007)

Method	Sample	Sample preparation	Recovery ± standard deviation	LOQ	References
LC-MS/MS	Surface waters and WWTP influents and effluents	SPE	72 ± 16% - 99 ± 3%	0.8 – 8 ng L^{-1}	(Gros et al., 2009)
LC-MS/MS	Surface waters close to effluent discharge	SPE	83.2 ± 3.1% - 92.2 ± 4.9%	0.11 ng L^{-1}	(Madureira et al., 2009)
UPLC - MS/MS	Surface waters	SPE	99.3 ± 2.2%	2.85 ng L^{-1}	(Conley et al., 2008)
UPLC - MS/MS	WWTP influents and effluents	SPE	71 ± 3%	1.5 ng L^{-1}	(Huerta-Fontela et al., 2010)
ELISA	Surface water	None	- (validated by LC-MS/MS)	0.05 – 50 µg L^{-1} (quantitation range)	(Bahlmann et al., 2009)
			FLUOXETINE		
LC-APCI-MS/MS	Fish tissues (n=8)	PLE followed by SPE	96.2 ± 2.9%	0.07 ng g^{-1}	(Chu and Metcalfe, 2007)
LC-(ESI)-MS/MS	Surface waters	SPE	105 ± 6% (high spiking level); 74 ± 12% (low spiking level)	76 ng L^{-1}	(Gros et al., 2006)
LC-(ESI)-MS/MS	WWTP effluent	SPE	60 ± 2% (high spiking level); 74 ± 2% (low spiking level)	70 ng L^{-1}	(Gros et al., 2006)
LC-(ESI)-MS/MS	WWTP influent	SPE	108 ± 4% (high spiking level); 67 ± 12% (low spiking level)	100 ng L^{-1}	(Gros et al., 2006)
HPLC-(ESI)-MS	STP influents and effluents	SPE and LLE	64 – 92% (different samples)	120 pg L^{-1}	(Vasskog et al., 2006)
LC-MS/MS	Surface waters and WWTP influents and effluents	SPE	70 ± 6% - 112 ± 8%	0.8 – 5 ng L^{-1}	(Gros et al., 2009)
UPLC - MS/MS	Surface waters	SPE	80.1 ± 3.6%	3.29 ng L^{-1}	(Conley et al., 2008)
UPLC - MS/MS	WWTP influents and effluents	SPE	62 ± 4%	15 ng L^{-1}	(Huerta-Fontela et al., 2010)

Method	Sample	Sample preparation	Recovery ± standard deviation	LOQ	References
UPLC – MS/MS	Surface waters and WWTP influents and effluents	SPE	53 ± 17% - 74 ± 5% (SPE absolute recovery); 88 ± 12% - 104 ± 14% (SPE relative recovery)	3.0 – 5.0 ng L^{-1}	(Baker and Kasprzyk-Hordern, 2011)
PAROXETINE					
LC-APCI-MS/MS	Fish tissues (n=8)	PLE followed by SPE	99.2 ± 3.5%	0.24 ng g^{-1}	(Chu and Metcalfe, 2007)
LC-(ESI)-MS/MS	Surface waters	SPE	95 ± 5% (high spiking level); 110 ± 12% (low spiking level)	20 ng L^{-1}	(Gros et al., 2006)
LC-(ESI)-MS/MS	WWTP effluent	SPE	65 ± 3% (high spiking level); 76 ± 12% (low spiking level)	26 ng L^{-1}	(Gros et al., 2006)
LC-(ESI)-MS/MS	WWTP influent	SPE	96 ± 1% (high spiking level); 84 ± 4% (low spiking level)	22 ng L^{-1}	(Gros et al., 2006)
HPLC-(ESI)-MS	STP influents and effluents	SPE and LLE	71 – 92% (different samples)	120 pg L^{-1}	(Vasskog et al., 2006)
LC-MS/MS	Surface waters and WWTP influents and effluents	SPE	84 ± 19% - 99 ± 3%	2 – 3 ng L^{-1}	(Gros et al., 2009)
UHPLC-MS/MS	Surface waters and WWTP effluents	SPE	94 ± 9% - 97 ± 9% (surface water); 91 ± 5% - 92 ± 3% (WWTP effluents)	0.2 ng L^{-1} and 3.6 ng L^{-1}	(Gracia-Lor et al., 2010)
VENLAFAXINE					
UHPLC-MS/MS	Surface waters and WWTP effluents	SPE	70 ± 8% - 110 ± 2% (surface water); 91 ± 4% - 88 ± 12% (WWTP effluents)	19 ng L^{-1} and 43 ng L^{-1}	(Gracia-Lor et al., 2010)
UPLC – MS/MS	WWTP influents and effluents	SPE	72 ± 4%	1.5 ng L^{-1}	(Huerta-Fontela et al., 2010)
UPLC – MS/MS	Surface waters and WWTP influents and effluents	SPE	85 ± 8% - 97 ± 8% (SPE absolute recovery); 90 ± 6% - 113 ± 7% (SPE relative recovery)	0.5 – 3.6 ng L^{-1}	(Baker and Kasprzyk-Hordern, 2011)

Method	Sample	Sample preparation	Recovery ± standard deviation	LOQ	References
			CITALOPRAM		
HPLC-(ESI)-MS	STP influents and effluents	SPE and LLE	36 – 72% (different samples)	160 pg L^{-1}	(Vasskog et al., 2006)
			FLUVOXAMINE		
HPLC-(ESI)-MS	STP influents and effluents	SPE and LLE	56 – 82% (different samples)	150 pg L^{-1}	(Vasskog et al., 2006)
			SETRALINE		
HPLC-(ESI)-MS	STP influents and effluents	SPE and LLE	52 – 85% (different samples)	290 pg L^{-1}	(Vasskog et al., 2006)
UPLC - MS/MS	Surface waters	SPE	80.6 ± 3.0%	1.92 ng L^{-1}	(Conley et al., 2008)
			NORFLUOXETINE		
LC-APCI-MS/MS	Fish tissues (n=8)	PLE followed by SPE	85.6 ± 4.2%	0.14 ng g^{-1}	(Chu and Metcalfe, 2007)
UPLC - MS/MS	Surface waters	SPE	71.5 ± 4.7%	1.84 ng L^{-1}	(Conley et al., 2008)
			RISPERIDONE		
UHPLC-MS/MS	Surface waters and WWTP effluents	SPE	76 ± 11% - 88 ± 6% (surface water); 91 ± 4% - 114 ± 9% (WWTP effluents)	2.0 ng L^{-1} and 5.9 ng L^{-1}	(Gracia-Lor et al., 2010)

2.8 CONCLUDING REMARKS

The literature data here presented constitute a clear evidence of the widespread occurrence of psychiatric pharmaceuticals in the environment.

The excretion of the unchanged or conjugated forms by the human body, along with the inadequacy of WWTPs removal methods represent the major pathway of entrance of psychiatric pharmaceuticals into the environment. Some of the most commonly used methods in wastewater treatment have removal efficiencies below 10% and thus, large amounts of psychiatric pharmaceuticals pass through WWTPs completely unaffected. In the last 20 years there has been an increasing effort to improve the efficiency of WWTPs and to develop new remediation strategies. However, these measures were only partially successful and until now, it has not been found a viable alternative to overcome the continuous introduction of these compounds into the environment. Ultimately, groundwater, which constitutes the largest reservoir of potable water on the planet, might be affected as reported by several studies which have already identified these pollutants in groundwater samples. Widespread contamination also affects water reuse for agricultural purposes and drinking water resources. As a result, one of the main focuses of future investigation should comprise prevention of continuous pollution and feasible remediation strategies.

Nowadays, mainly due to major advances in the development of powerful analytical methods, there is a considerable knowledge on the occurrence of psychiatric pharmaceuticals (and other pharmaceuticals, in general) in several environmental compartments. However, conclusive data about the persistence, fate and ecotoxicological effects of psychiatric pharmaceuticals are still missing. Very few data is available on the impact of environmental transformations (such as biodegradation and photodegradation) in the fate and persistence of these compounds. Moreover, for the majority of the referred pharmaceuticals, the identity and relevance of the photoproducts generated in the environment remain completely unknown. Comprehensive approaches to this issue should, therefore, consider that environmental by-products might also be relevant environmental pollutants. Concerning the ecotoxicological importance of these pharmaceuticals, there are some evidences that compounds such as diazepam, carbamazepine and fluoxetine are potentially being accumulated in aquatic organisms. The study of life cycle toxicity, as well as the investigation of the adverse effects caused by the exposure to complex mixtures of psychiatric pharmaceuticals with the same mode of action, should also be considered a strong topic to be addressed in a near future.

In conclusion, this literature review allows a clear identification of the main research needs in this field of concern. The persistence of benzodiazepines (anxiolytic pharmaceuticals) in aquatic environments was recognized as a poorly studied topic. In line with this assessment, part of the work developed and presented in this thesis is intended to give a significant contribution to better understand this subject. Data presented in this chapter also indicate that carbamazepine is a potentially adequate marker of anthropogenic pollution. A better knowledge of the environmental behavior of this pharmaceutical might constitute a valuable tool for future investigation. Thus, this work also aims to clarify the persistence and fate of carbamazepine (namely, photoreactivity and partition at water/soil interfaces) as well as to develop a fast and inexpensive screening methodology which is of the utmost importance for large environmental screenings.

2.9 REFERENCES

Andreozzi, R., Raffaele, M., Nicklas, P., **2003**. Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environment. *Chemosphere* 50, 1319-1330.

Andreozzi, R., Raffaele, M., Pinto, G., Pollio, A., **2002**. Carbamazepine in water: persistence in the environment, ozonation treatment and preliminary assessment on algal toxicity. *Water Res*. 36, 2869-2877.

Ariffin, M.M., Miller, E.I., Cormack, P.A., Anderson, R.A., **2007**. Molecularly imprinted solid-phase extraction of diazepam and its metabolites from hair samples. *Anal. Chem.* 79, 256-262.

Ashton, D., Hilton, M., Thomas, K.V., **2004**. Investigating the environmental transport of human pharmaceuticals to streams in the United Kingdom. *Sci. Total Environ*. 333, 167-184.

Bahlmann, A., Weller, M.G., Panne, U., Schneider, R.J., **2009**. Monitoring carbamazepine in surface and wastewaters by an immunoassay based on a monoclonal antibody. *Anal. Bioanal. Chem*. 395, 1809-1820.

Baker, D.R., Kasprzyk-Hordern, B., **2011**. Multi-residue analysis of drugs of abuse in wastewater and surface water by solid-phase extraction and liquid chromatography-positive electrospray ionisation tandem mass spectrometry. *J. Chromatogr. A* 1218, 1620-1631.

Bencan, Z., Sledge, D., Levin, E.D., **2009**. Buspirone, chlordiazepoxide and diazepam effects in a zebrafish model of anxiety. *Pharmacol. Biochem. Be.* 94, 75-80.

Benotti, M.J., Trenholm, R.A., Vanderford, B.J., Holady, J.C., Stanford, B.D., Snyder, S.A., **2009**. Pharmaceuticals and endocrine disrupting compounds in US drinking water. *Environ. Sci. Technol.* 43, 597-603.

Boreen, A.L., Arnold, W.A., McNeill, K., **2003**. Photodegradation of pharmaceuticals in the aquatic environment: a review. *Aquat. Sci.* 65, 320-341.

Bound, J.P., Kitsou, K., Voulvoulis, N., **2006**. Household disposal of pharmaceuticals and perception of risk to the environment. *Environ. Toxicol. Phar.* 21, 301-307.

Bringolf, R.B., Heltsley, R.M., Newton, T.J., Eads, C.B., Fraley, S.J., Shea, D., Cope, W.G., **2010**. Environmental occurrence and reproductive effects of the pharmaceutical fluoxetine in native freshwater mussels. *Environ. Toxicol. Chem.* 29, 1311-1318.

Brooks, B.W., Chambliss, C.K., Stanley, J.K., Ramirez, A., Banks, K.E., Johnson, R.D., Lewis, R.J., **2005**. Determination of select antidepressants in fish from an effluent-dominated stream. *Environ. Toxicol. Chem.* 24, 464-469.

Brooks, B.W., Foran, C.M., Richards, S.M., Weston, J., Turner, P.K., Stanley, J.K., Solomon, K.R., Slattery, M., La Point, T.W., **2003**a. Aquatic ecotoxicology of fluoxetine. *Toxicol. Lett.* 142, 169-183.

Brooks, B.W., Turner, P.K., Stanley, J.K., Weston, J.J., Glidewell, E.A., Foran, C.M., Slattery, M., La Point, T.W., Huggett, D.B., **2003**b. Waterborne and sediment toxicity of fluoxetine to select organisms. *Chemosphere* 52, 135-142.

Brunton, L.L., Parker, K.L., Blumenthal, D. K., Buxton, I.L.O. (Ed.), **2008**. *Goodman & Gilman's Manual of Pharmacology and Therapeutics*. McGraw-Hill, San Diego, California.

Buchberger, W.W., **2007**. Novel analytical procedures for screening of drug residues in water, waste water, sediment and sludge. *Anal. Chim. Acta* 593, 129-139.

Buchberger, W.W., **2011**. Current approaches to trace analysis of pharmaceuticals and personal care products in the environment. *J. Chromatogr. A* A 1218, 603-618.

Calamari, D., Zuccato, E., Castiglioni, S., Bagnati, R., Fanelli, R., **2003**. Strategic survey of therapeutic drugs in the rivers Po and Lambro in northern Italy. *Environ. Sci. Technol.* 37, 1241-1248.

Calderón-Preciado, D., Jiménez-Cartagena, C., Matamoros, V., Bayona, J.M., **2011**. Screening of 47 organic microcontaminants in agricultural irrigation waters and their soil loading. *Water Res.* 45, 221-231.

Caliman, F.A., Gavrilescu, M., **2009**. Pharmaceuticals, personal care products and endocrine disrupting agents in the environment – A Review. *CLEAN – Soil, Air, Water* 37, 277-303.

Calleja, M.C., Persoone, G., Geladi, P., **1994**. Comparative acute toxicity of the first 50 multicenter evaluation of in-vitro cytotoxicity chemicals to aquatic non-vertebrates. *Arch. Environ. Con. Tox.* 26, 69-78.

Caminada, D., Escher, C., Fent, K., **2006**. Cytotoxicity of pharmaceuticals found in aquatic systems: comparison of PLHC-1 and RTG-2 fish cell lines. *Aquat. Toxicol.* 79, 114-123.

Carballa, M., Omil, F., Lema, J.M., **2008**. Comparison of predicted and measured concentrations of selected pharmaceuticals, fragrances and hormones in Spanish sewage. *Chemosphere* 72, 1118-1123.

Carballa, M., Omil, F., Lema, J.M., Llompart, M., Garcia-Jares, C., Rodriguez, I., Gomez, M., Ternes, T., **2004**. Behavior of pharmaceuticals, cosmetics and hormones in a sewage treatment plant. *Water Res.* 38, 2918-2926.

Carlsson, C., Johansson, A.K., Alvan, G., Bergman, K., Kuhler, T., **2006**. Are pharmaceuticals potent environmental pollutants? Part I: environmental risk assessments of selected active pharmaceutical ingredients. *Sci. Total Environ.* 364, 67-87.

Chefetz, B., Mualem, T., Ben-Ari, J., **2008**. Sorption and mobility of pharmaceutical compounds in soil irrigated with reclaimed wastewater. *Chemosphere* 73, 1335-1343.

Chenxi, W., Spongberg, A.L., Witter, J.D., **2008**. Determination of the persistence of pharmaceuticals in biosolids using liquid-chromatography tandem mass spectrometry. *Chemosphere* 73, 511-518.

Chiba, K., Horii, H., Chiba, T., Kato, Y., Hirano, T., Ishizaki, T., **1995**. Development and preliminary application of high-performance liquid-chromatographic assay of urinary metabolites of diazepam in humans. *J. Chromatogr. B* 668, 77-84.

Chiron, S., Minero, C., Vione, D., **2006**. Photodegradation processes of the antiepileptic drug carbamazepine, relevant to estuarine waters. *Environ. Sci. Technol.* 40, 5977-5983.

Chouinard, G., Lefko-Singh, K., Teboul, E., **1999**. Metabolism of anxiolytics and hypnotics: benzodiazepines, buspirone, zoplicone, and zolpidem. *Cell. Mol. Neurobiol.* 19, 533-552.

Chu, S., Metcalfe, C.D., **2007**. Analysis of paroxetine, fluoxetine and norfluoxetine in fish tissues using pressurized liquid extraction, mixed mode solid phase extraction cleanup and liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A* 1163, 112-118.

Clara, M., Strenn, B., Kreuzinger, N., **2004**. Carbamazepine as a possible anthropogenic marker in the aquatic environment: investigations on the behaviour of carbamazepine in wastewater treatment and during groundwater infiltration. *Water Res.* 38, 947-954.

Coetsier, C.M., Spinelli, S., Lin, L., Roig, B., Touraud, E., **2009**. Discharge of pharmaceutical products (PPs) through a conventional biological sewage treatment plant: MECs vs PECs? *Environ. Int*. 35, 787-792.

Collier, A.C., **2007**. Pharmaceutical contaminants in potable water: Potential concerns for pregnant women and children. *EcoHealth* 4, 164-171.

Conkle, J.L., White, J.R., Metcalfe, C.D., **2008**. Reduction of pharmaceutically active compounds by a lagoon wetland wastewater treatment system in Southeast Louisiana. *Chemosphere* 73, 1741-1748.

Conley, J.M., Symes, S.J., Kindelberger, S.A., Richards, S.A., **2008**. Rapid liquid chromatography-tandem mass spectrometry method for the determination of a broad mixture of pharmaceuticals in surface water. *J. Chromatogr. A* 1185, 206-215.

Conners, D.E., Rogers, E.D., Armbrust, K.L., Kwon, J.-W., Black, M.C., **2009**. Growth and development of tadpoles (*Xenopus laevis*) exposed to selective serotonin reuptake inhibitors, fluoxetine and sertraline, throughout metamorphosis. *Environ. Toxicol. Chem.* 28, 2671-2676.

Courtheyn, D., Le Bizec, B., Brambilla, G., De Brabander, H.F., Cobbaert, E., de Wiele, A.V., Vercammen, J., De Wasch, K., **2002**. Recent developments in the use and abuse of growth promoters. *Anal. Chim. Acta* 473, 71-82.

Crane, M., Watts, C., Boucard, T., **2006**. Chronic aquatic environmental risks from exposure to human pharmaceuticals. *Sci. Total Environ.* 367, 23-41.

Cunningham, V.L., Buzby, M., Hutchinson, T. , Mastrocco, F. , Parke, N., Roden, N. , **2006**. Effects of human pharmaceuticals on aquatic life: Next steps. *Environ. Sci. Technol*. 40, 3456–3462.

Cunningham, V.L., Constable, D.J.C., Hannah, R.E., **2004**. Environmental risk assessment of paroxetine. *Environ. Sci. Technol.* 38, 3351-3359.

Debska, J., Kot-Wasik, A., Namiesnik, J., **2004**. Fate and analysis of pharmaceutical residues in the aquatic environment. *Cr. Rev. Anal. Chem.* 34, 51-67.

Doll, T.E., Frimmel, F.H., **2003**. Fate of pharmaceuticals - photodegradation by simulated solar UV-light. *Chemosphere* 52, 1757-1769.

Drewes, J.E., Heberer, T., Reddersen, K., **2001**. Fate of pharmaceuticals during indirect potable reuse. 2nd World Water Congress of the International-Water-Association, Berlin, Germany, pp. 73-80.

Eckel, W.P., Ross, B., and Isensee, R.K, **1993**. Pentobarbital found in groundwater. *Groundwater* 31, 801-804.

Ferrari, B., Paxéus, N., Giudice, R.L., Pollio, A., Garric, J., **2003**. Ecotoxicological impact of pharmaceuticals found in treated wastewaters: study of carbamazepine, clofibric acid, and diclofenac. *Ecotox. Environ. Safe.* 55, 359-370.

Fong, P.P., **1998**. Zebra mussel spawning is induced in low concentrations of putative serotonin reuptake inhibitors. *Biol. Bull.* 194, 143-149.

Fong, P.P., Huminski, P.T., D'Urso, L.M., **1998**. Induction and potentiation of parturition in fingernail clams (*Sphaerium striatinum*) by selective serotonin re-uptake inhibitors (SSRIs). *J. Exp. Zool.* 280, 260-264.

Fong, P.P., Molnar, N., **2008**. Norfluoxetine induces spawning and parturition in estuarine and freshwater bivalves. *Bull. Environ. Contam. Toxicol*. 81, 535-538

Foran, C.M., Weston, J., Slattery, M., Brooks, B.W., Huggett, D.B., **2004**. Reproductive assessment of Japanese medaka (*Oryzias latipes*) following a four-week fluoxetine (SSRI) exposure. *Arch. Environ. Con. Tox.* 46, 511-517.

Foster, H.R., Burton, G.A., Basu, N., Werner, E.E., **2010**. Chronic exposure to fluoxetine (prozac) causes developmental delays in *Rana pipiens* larvae. *Environ. Toxicol. Chem*. 29, 2845-2850.

Gaskins, L.A., Massey, J.G., Ziccardi, M.H., **2008**. Effect of oral diazepam on feeding behavior and activity of Hawai'i 'amakihi (*Hemignathus virens*). *Appl. Anim. Behav. Sci*. 112, 384-394.

Ghasemi, J., Niazi, A., Ghorbani, R., **2006**. Determination of trace amounts of lorazepam by adsorptive cathodic differential pulse stripping method in pharmaceutical formulations and biological fluids. *Anal. Lett.* 39, 1159-1169.

Gielen, G.J.H.P., Heuvel, M.R.v.d., Clinton, P.W., Greenfield, L.G., **2009**. Factors impacting on pharmaceutical leaching following sewage application to land. *Chemosphere* 74, 537-542.

Glassmeyer, S., Kolpin, D.W., Furlong, E.T., Focazio, M.T., **2008**. Environmental presence and persistence of pharmaceuticals: an overview. in: Aga, D.S. (Ed.). *Fate of Pharmaceuticals in the Environment and in Water Treatment Systems*. CRC Press, Taylor & Francis Books, Boca Raton, pp. 3-51.

Gómez, M.J., Martínez Bueno, M.J., Lacorte, S., Fernández-Alba, A.R., Agüera, A., **2007**. Pilot survey monitoring pharmaceuticals and related compounds in a sewage treatment plant located on the Mediterranean coast. *Chemosphere* 66, 993-1002.

González Alonso, S., Catalá, M., Maroto, R.R., Gil, J.L.R., de Miguel, Á.G., Valcárcel, Y., **2010**. Pollution by psychoactive pharmaceuticals in the Rivers of Madrid metropolitan area (Spain). *Environ. Int.* 36, 195-201.

Gracia-Lor, E., Sancho, J.V., Hernández, F., **2010**. Simultaneous determination of acidic, neutral and basic pharmaceuticals in urban wastewater by ultra high-pressure liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A* 1217, 622-632.

Gracia-Lor, E., Sancho, J.V., Hernández, F., **2011**. Multi-class determination of around 50 pharmaceuticals, including 26 antibiotics, in environmental and wastewater samples by ultra-high performance liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A* 1218, 2264-2275.

Gros, M., Petrovic, M., Barceló, D., **2006**. Development of a multi-residue analytical methodology based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) for screening and trace level determination of pharmaceuticals in surface and wastewaters. *Talanta* 70, 678-690.

Gros, M., Petrović, M., Barceló, D., **2009**. Tracing pharmaceutical residues of different therapeutic classes in environmental waters by using liquid chromatography/quadrupole-linear ion trap mass spectrometry and automated library searching. *Anal. Chem*. 81, 898-912.

Gros, M., Petrovic, M., Ginebreda, A., Barceló, D., **2010**. Removal of pharmaceuticals during wastewater treatment and environmental risk assessment using hazard indexes. *Environ. Int.* 36, 15-26.

Guler, Y., Ford, A.T., **2010**. Anti-depressants make amphipods see the light. *Aquat. Toxicol.* 99, 397-404.

Gust, M., Buronfosse, T., Giamberini, L., Ramil, M., Mons, R., Garric, J., **2009**. Effects of fluoxetine on the reproduction of two prosobranch mollusks: *Potamopyrgus antipodarum* and *Valvata piscinalis*. *Environ. Pollut*. 157, 423-429.

Halling-Sorensen, B., Nielsen, S.N., Lanzky, P.F., Ingerslev, F., Holten Lutzhoft, H.C., Jorgensen, S.E., **1998**. Occurrence, fate and effects of pharmaceutical substances in the environment – a review. *Chemosphere* 36, 357-393.

Hanna, M., de Biasi, V., Bond, B., Salter, C., Hutt, A.J., Camilleri, P., **1998**. Estimation of the partitioning characteristics of drugs: a comparison of a large and diverse drug series utilizing chromatographic and electrophoretic methodology. *Anal. Chem.* 70, 2092-2099.

Health, N., **2011**. The internet drug index (the top 200 prescriptions for 2009 by number dispensed). http://www.rxlist.com (Accessed January 2011).

Heberer, T., **2002**. Tracking persistent pharmaceutical residues from municipal sewage to drinking water. *J. Hydrol.* 266, 175-189.

Henry, T.B., Black, M.C., **2008**. Acute and chronic toxicity of fluoxetine (selective serotonin reuptake inhibitor) in western mosquitofish. *Arch. Environ. Con. Toxicol.* 54, 325-330.

Henry, T.B., Kwon, J.W., Armbrust, K.L., Black, M.C., **2004**. Acute and chronic toxicity of five selective serotonin reuptake inhibitors in *Ceriodaphnia dubia*. *Environ. Toxicol. and Chem.* 23, 2229-2233.

Holm, J.V., Rugge, K., Bjerg, P.L., Christensen, T.H., **1995**. Occurrence and distribution of pharmaceutical organic-compounds in the groundwater downgradient of a Landfill (Grindsted, Denmark). *Environ. Sci. Technol*. 29, 1415-1420.

Huerta-Fontela, M., Galceran, M.T., Ventura, F., **2010**. Fast liquid chromatography-quadrupole-linear ion trap mass spectrometry for the analysis of pharmaceuticals and hormones in water resources. *J. Chromatogr. A* 1217, 4212-4222.

Huo, S.M., Yang, H., Deng, A.P., **2007**. Development and validation of a highly sensitive ELISA for the determination of pharmaceutical indomethacin in water samples. *Talanta* 73, 380-386.

International Narcotics Control Board, **2010**. *Psychotropic Substances - Report 2009: Statistics for 2008*. New York.

Jelic, A., Gros, M., Ginebreda, A., Cespedes-Sánchez, R., Ventura, F., Petrovic, M., Barcelo, D., **2011**. Occurrence, partition and removal of pharmaceuticals in sewage water and sludge during wastewater treatment. *Water Res*. 45, 1165-1176.

Jjemba, P.K., **2006**. Excretion and ecotoxicity of pharmaceutical and personal care products in the environment. *Ecotoxicol. Environ. Safe.* 63, 113-130.

Johnson, D.J., Sanderson, H., Brain, R.A., Wilson, C.J., Solomon, K.R., **2007**. Toxicity and hazard of selective serotonin reuptake inhibitor antidepressants fluoxetine, fluvoxamine, and sertraline to algae. *Ecotoxicol. Environ. Safe.* 67, 128-139.

Jones, O.A.H., Lester, J.N., Voulvoulis, N., **2005**a. Pharmaceuticals: a threat to drinking water? *Trends Biotechnol.* 23, 163-167.

Jones, O.A.H., Voulvoulis, N., Lester, J.N., **2001**. Human pharmaceuticals in the aquatic environment - A review. *Environ. Technol.* 22, 1383-1394.

Jones, O.A.H., Voulvoulis, N., Lester, J.N., **2005**b. Human pharmaceuticals in wastewater treatment processes. *Crit. Rev. Env. Sci. Tec.* 35, 401-427.

Jos, A., Repetto, G., Rios, J.C., Hazen, M.J., Molero, M.L., del Peso, A., Salguero, M., Fernández-Freire, P., Pérez-MartIn, J.M., Cameán, A., **2003**. Ecotoxicological evaluation of carbamazepine using six different model systems with eighteen endpoints. *Toxicol. in Vitro* 17, 525-532.

Joss, A., Zabczynski, S., Gobel, A., Hoffmann, B., Loffler, D., McArdell, C.S., Ternes, T.A., Thomsen, A., Siegrist, H., **2006**. Biological degradation of pharmaceuticals in municipal wastewater treatment: Proposing a classification scheme. *Water Res*. 40, 1686-1696.

Kar, A., **2007**. *Medicinal Chemistry*, 4th ed. New age international publishers, New Delhi, India.

Kasprzyk-Hordern, B., Dinsdale, R.M., Guwy, A.J., **2008**. The occurrence of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs in surface water in South Wales, UK. *Water Res.* 42, 3498-3518.

Kinney, C.A., Furlong, E.T., Zaugg, S.D., Burkhard, M.R., Werner, S.L., Cahill, J.D., Jorgensen, G.R., **2006**. Survey of organic wastewater contaminants in biosolids destined for land application. *Environ. Sci. Technol*. 40, 7207-7215.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., **2002**. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: A national reconnaissance. *Environ. Sci. Technol.* 36, 1202-1211.

Kosjek, T., Heath, E., **2010**. Tools for evaluating selective serotonin re-uptake inhibitor residues as environmental contaminants. *TrAC - Trends Anal. Chem*. 29, 832-847.

Kostopoulou, M., Nikolaou, A., **2008**. Analytical problems and the need for sample preparation in the determination of pharmaceuticals and their metabolites in aqueous environmental matrices. *TrAC - Trends Anal. Chem.* 27, 1023-1035.

Kot-Wasik, A., Debska, J., Namiesnik, J., **2007**. Analytical techniques in studies of the environmental fate of pharmaceuticals and personal-care products. *TrAC - Trends Anal. Chem*. 26, 557-568.

Kummerer, K. (Ed.), **2008**. *Pharmaceuticals in the Environment: Sources, Fate, Effects and Risks*. Springer-Verlag, Berlin.

Kwon, J.-W., Armbrust, K.L., **2005**. Degradation of citalopram by simulated sunlight. *Environ. Toxicol. Chem.* 24, 1618-1623.

Kwon, J.W., Armbrust, K.L., **2006**. Laboratory persistence and fate of fluoxetine in aquatic environments. *Environ. Toxicol. Chem.* 25, 2561-2568.

Kwon, J.W., Armbrust, K.L., **2008**. Aqueous solubility, n-octanol-water partition coefficient, and sorption of five selective serotonin reuptake inhibitors to sediments and soils. *Bull. Environ. Contam. Toxicol.* 81, 128-135.

Kwon, J.W., Armbrust, K.L., Bay, S.M., Vidal-Dorsch, D., Xia, K., **2009**. Determination of 17α-Ethynylestradiol, Carbamazepine, Diazepam, Simvastatin, and Oxybenzone in Fish Livers. *J. AOAC Int.* 92, 359-369.

Lam, M.W., Mabury, S.A., **2005**. Photodegradation of the pharmaceuticals atorvastatin, carbamazepine, levofloxacin, and sulfamethoxazole in natural waters. *Aquat. Sci.* 67, 177-188.

Lam, M.W., Young, C.J., Mabury, S.A., **2005**. Aqueous photochemical reaction kinetics and transformations of fluoxetine. *Environ. Sci. Technol.* 39, 513-522.

Lapen, D.R., Topp, E., Metcalfe, C.D., Li, H., Edwards, M., Gottschall, N., Bolton, P., Curnoe, W., Payne, M., Beck, A., **2008**. Pharmaceutical and personal care products in tile drainage following land application of municipal biosolids. *Sci. Total Environ.* 399, 50-65.

Leif, W., Inka, H., Matthias, E., Heinz, H., **2004**. Impact of leaky sewers on groundwater quality. *Acta Hydroch. Hydrob.* 32, 361-373.

Li, Z.-H., Li, P., Rodina, M., Randak, T., **2010**. Effect of human pharmaceutical carbamazepine on the quality parameters and oxidative stress in common carp (*Cyprinus carpio L.*) spermatozoa. *Chemosphere* 80, 530-534.

Li, Z.-H., Zlabek, V., Velisek, J., Grabic, R., Machova, J., Kolarova, J., Li, P., Randak, T., **2011**. Acute toxicity of carbamazepine to juvenile rainbow trout (*Oncorhynchus mykiss*): Effects on antioxidant responses, hematological parameters and hepatic EROD. *Ecotoxicol. Environ. Safe.* 74, 319-327.

Lilius, H., Hastbacka, T., Isomaa, B., **1995**. A comparison of the toxicity of 30 reference chemicals to *Daphnia magna* and *Daphnia pulex*. *Environ. Toxicol. Chem.* 14, 2085-2088.

Loffler, D., Rombke, J., Meller, M., Ternes, T.A., **2005**. Environmental fate of pharmaceuticals in water/sediment systems. *Environ. Sci. Technol.* 39, 5209-5218.

Loganathan, B., Phillips, M., Mowery, H., Jones-Lepp, T.L., **2009**. Contamination profiles and mass loadings of macrolide antibiotics and illicit drugs from a small urban wastewater treatment plant. *Chemosphere* 75, 70-77.

Loos, R., Gawlik, B.M., Locoro, G., Rimaviciute, E., Contini, S., Bidoglio, G., **2009**. EU-wide survey of polar organic persistent pollutants in European river waters. *Environ. Poll.* 157, 561-568.

Loos, R., Locoro, G., Comero, S., Contini, S., Schwesig, D., Werres, F., Balsaa, P., Gans, O., Weiss, S., Blaha, L., Bolchi, M., Gawlik, B.M., **2010**. Pan-European survey on the occurrence of selected polar organic persistent pollutants in ground water. *Water Res*. 44, 4115-4126.

López-Serna, R., Pérez, S., Ginebreda, A., Petrovic, M., Barceló, D., **2010**. Fully automated determination of 74 pharmaceuticals in environmental and waste waters by online solid phase extraction-liquid chromatography-electrospray-tandem mass spectrometry. *Talanta* 83, 410-424.

Madureira, T.V., Barreiro, J.C., Rocha, M.J., Cass, Q.B., Tiritan, M.E., **2009**. Pharmaceutical trace analysis in aqueous environmental matrices by liquid chromatography-ion trap tandem mass spectrometry. *J. Chromatogr. A* 1216, 7033-7042.

Matamoros, V., Duhec, A., Albaiges, J., Bayona, J.M., **2009**. Photodegradation of carbamazepine, ibuprofen, ketoprofen and 17α - ethinylestradiol in fresh and seawater. *Water Air Soil Poll.* 196, 161-168.

Metcalfe, C.D., Miao, X.-S., Koenig, B.G., Struger, J., **2003**. Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower Great Lakes, Canada. *Environ. Toxicol. Chem.* 22, 2881-2889.

Mills, D.S., **2003**. Medical paradigms for the study of problem behaviour: a critical review. *Appl. Anim. Behav. Sci.* 81, 265-277.

Moffat, A.C., Osselton, M.D., Widdop, B., Galichet, L.Y., **2005**. *Clarke's Analysis of Drugs and Poisons*, 3rd edition. Pharmaceutical Press, London.

Moldovan, Z., **2006**. Occurrences of pharmaceutical and personal care products as micropollutants in rivers from Romania. *Chemosphere* 64, 1808-1817.

Nakamura, Y., Yamamoto, H., Sekizawa, J., Kondo, T., Hirai, N., Tatarazako, N., **2008**. The effects of pH on fluoxetine in Japanese medaka (*Oryzias latipes*): Acute toxicity in fish larvae and bioaccumulation in juvenile fish. *Chemosphere* 70, 865-873.

Nassef, M., Matsumoto, S., Seki, M., Khalil, F., Kang, I.J., Shimasaki, Y., Oshima, Y., Honjo, T., **2010**. Acute effects of triclosan, diclofenac and carbamazepine on feeding performance of Japanese medaka fish (*Oryzias latipes*). *Chemosphere* 80, 1095-1100.

Nunes, B., Carvalho, F., Guilhermino, L., **2005**. Acute toxicity of widely used pharmaceuticals in aquatic species: *Gambusia holbrooki*, *Artemia parthenogenetica* and *Tetraselmis chuii*. *Ecotoxicol. Environ. Safe*. 61, 413-419.

Oggier, D.M., Weisbrod, C.J., Stoller, A.M., Zenker, A.K., Fent, K., **2010**. Effects of diazepam on gene expression and link to physiological effects in different life stages in zebrafish *Danio rerio*. *Environ. Sci. Technol.* 44, 7685-7691.

Pal, A., Gin, K.Y.-H., Lin, A.Y.-C., Reinhard, M., **2010**. Impacts of emerging organic contaminants on freshwater resources: Review of recent occurrences, sources, fate and effects. *Sci. Total Environ*. 408, 6062-6069.

Paranychianakis, N.V., Angelakis, A.N., Leverenz, H., Tchobanoglous, G., **2006**. Treatment of wastewater with slow rate systems: A review of treatment processes and plant functions. *Cr. Rev. Environ. Sci. Technol.* 36, 187-259.

Pascoe, D., Karntanut, W., Muller, C.T., **2003**. Do pharmaceuticals affect freshwater invertebrates? A study with the cnidarian *Hydra vulgaris*. *Chemosphere* 51, 521-528.

Paterson, G., Metcalfe, C.D., **2008**. Uptake and depuration of the anti-depressant fluoxetine by the Japanese medaka (*Oryzias latipes*). *Chemosphere* 74, 125-130.

Pavlic, M., Libiseller, K., Grubwieser, P., Schubert, H., Rabl, W., **2007**. Medicolegal aspects of tetrazepam metabolism. *Int. J. Legal Med.* 121, 169-174.

Pedersen, J.A., Soliman, M., Suffet, I.H., **2005**. Human pharmaceuticals, hormones, and personal care product ingredients in runoff from agricultural fields irrigated with treated wastewater. *J. Agr. Food Chem.* 53, 1625-1632.

Péry, A.R.R., Gust, M., Vollat, B., Mons, R., Ramil, M., Fink, G., Ternes, T., Garric, J., **2008**. Fluoxetine effects assessment on the life cycle of aquatic invertebrates. *Chemosphere* 73, 300-304.

Peschka, M., Eubeler, J.P., Knepper, T.P., **2006**. Occurrence and fate of barbiturates in the aquatic environment. *Environ. Sci. Technol.* 40, 7200-7206.

Petrovic, M., Barceló, D. (Eds.), **2007**. *Analysis, Fate and Removal of Pharmaceuticals in the Water Cycle*. Elsevier, Amsterdam, The Netherlands.

Pomati, F., Orlandi, C., Clerici, M., Luciani, F., Zuccato, E., **2008**. Effects and interactions in an environmentally relevant mixture of pharmaceuticals. *Toxicol. Sci.* 102, 129-137.

Quinn, B., Gagne, F., Blaise, C., **2004**. Oxidative metabolism activity in *Hydra attenuata* exposed to carbamazepine. *Fresen. Environ. Bull.* 13, 783-788.

Quinn, B., Gagné, F., Blaise, C., **2009**. Evaluation of the acute, chronic and teratogenic effects of a mixture of eleven pharmaceuticals on the cnidarian, *Hydra attenuata*. *Sci. Total Environ.* 407, 1072-1079.

Rabiet, M., Togola, A., Brissaud, F., Seidel, J.L., Budzinski, H., Elbaz-Poulichet, F., **2006**. Consequences of treated water recycling as regards pharmaceuticals and drugs in surface and ground waters of a medium-sized Mediterranean catchment. *Environ. Sci. Technol.* 40, 5282-5288.

Ramirez, A.J., Mottaleb, M.A., Brooks, B.W., Chambliss, C.K., **2007**. Analysis of pharmaceuticals in fish using liquid chromatography-tandem mass spectrometry. *Anal. Chem.* 79, 3155-3163.

Rao, N., **2007**. The clinical pharmacokinetics of escitalopram. *Clin. Pharmacokinet*. 46, 281-290.

Redshaw, C.H., Cooke, M.P., Talbot, H.M., McGrath, S., Rowland, S.J., **2008**. Low biodegradability of fluoxetine HCl, diazepam and their human metabolites in sewage sludge-amended soil. *J. Soils Sediments* 8, 217-230.

Richards, S.M., Cole, S.E., **2006**. A toxicity and hazard assessment of fourteen pharmaceuticals to *Xenopus laevis* larvae. *Ecotoxicology* 15, 647-656.

Sabljic, A., Güsten, H., Verhaar, H., Hermens, J., **1995**. QSAR modelling of soil sorption. Improvements and systematics of log K_{OC} vs. log K_{OW} correlations. *Chemosphere* 31, 4489-4514.

Sacher, F., Lang, F.T., Brauch, H.J., Blankenhorn, I., **2001**. Pharmaceuticals in groundwaters - Analytical methods and results of a monitoring program in Baden-Wurttemberg, Germany. *J. Chromatogr. A* 938, 199-210.

Sánchez-Argüello, P., Fernández, C., Tarazona, J.V., **2009**. Assessing the effects of fluoxetine on *Physa acuta* (Gastropoda, Pulmonata) and *Chironomus riparius* (Insecta, Diptera) using a two-species water-sediment test. *Sci. Total Environ.* 407, 1937-1946.

Sanderson, H., Johnson, D.J., Wilson, C.J., Brain, R.A., Solomon, K.R., **2003**. Probabilistic hazard assessment of environmentally occurring pharmaceuticals toxicity to fish, daphnids and algae by ECOSAR screening. *Toxicol. Lett.* 144, 383-395.

Scheytt, T., Mersmann, P., Lindstädt, R., Heberer, T., **2005**. Determination of sorption coefficients of pharmaceutically active substances carbamazepine, diclofenac, and ibuprofen, in sandy sediments. *Chemosphere* 60, 245-253.

Schnell, S., Bols, N.C., Barata, C., Porte, C., **2009**. Single and combined toxicity of pharmaceuticals and personal care products (PPCPs) on the rainbow trout liver cell line RTL-W1. *Aquat. Toxicol.* 93, 244-252.

Schultz, M.M., Furlong, E.T., **2008**. Trace analysis of antidepressant pharmaceuticals and their select degradates in aquatic matrixes by LC/ESI/MS/MS. *Anal. Chem.* 80, 1756-1762.

Shelver, W.L., Shappell, N.W., Franek, M., Rubio, F.R., **2008**. ELISA for sulfonamides and its application for screening in water contamination. *J. Agric. Food Chem.* 56, 6609-6615.

Shon, H.K., Vigneswaran, S., Snyder, S.A., **2006**. Effluent organic matter (EfOM) in wastewater: Constituents, effects, and treatment. *Cr. Rev. Env. Sci. Tec.* 36, 327-374.

Sliwka-Kaszynska, M., Kot-Wasik, A., Namiesnik, J., **2003**. Preservation and storage of water samples. *Cr. Rev. Env. Sci. Tec.* 33, 31-44.

Smith-Kielland, A., Skuterud, B., Olsen, K.M., Morland, J., **2001**. Urinary excretion of diazepam metabolites in healthy volunteers and drug users. *Scand. J. Clin. Lab. Inv.* 61, 237-246.

Snyder, S.A., **2008**. Occurrence, treatment, and toxicological relevance of EDCs and pharmaceuticals in water. *Ozone-Sci. Eng.* 30, 65-69.

Snyder, S.A., Kelly, K.L., Grange, A.H., Sovocool, G.W., Snyder, E.M., Giesy, J.P., **2001**. Pharmaceuticals and Personal Care Products in the Waters of Lake Mead, Nevada, in: *Pharmaceuticals and Care Products in the Environment*. American Chemical Society, Washington, DC, pp. 116-139.

Stamatelatou, K., Frouda, C., Fountoulakis, M.S., Drillia, P., Kornaros, M., Lyberatos, G., **2003**. Pharmaceuticals and Health Care Products in Wastewater Effluents: the Example of Carbamazepine. in: Angelakis, A.N., Paranychianakis, N.V., Tsagarakis, K.P. (Eds.). *Water Recycling in the Mediterranean Region*, pp. 131-137.

Stanley, J.K., Ramirez, A.J., Chambliss, C.K., Brooks, B.W., **2007**. Enantiospecific sublethal effects of the antidepressant fluoxetine to a model aquatic vertebrate and invertebrate. *Chemosphere* 69, 9-16.

Stein, K., Ramil, M., Fink, G., Sander, M., Ternes, T.A., **2008**. Analysis and sorption of psychoactive drugs onto sediment. *Environ. Sci. Technol.* 42, 6415-6423.

Styrishave, B., Halling-Sørensen, B., Ingerslev, F., **2011**. Environmental risk assessment of three selective serotonin reuptake inhibitors in the aquatic environment: A case study including a cocktail scenario. *Environ. Toxicol. Chem.* 30, 254-261.

Ternes, T., Bonerz, M., Schmidt, T., **2001**. Determination of neutral pharmaceuticals in wastewater and rivers by liquid chromatography-electrospray tandem mass spectrometry. *J. Chromatogr. A* 938, 175-185.

Ternes, T.A., **1998**. Occurrence of drugs in German sewage treatment plants and rivers. *Water Res.* 32, 3245-3260.

Ternes, T.A., **2001**. Analytical methods for the determination of pharmaceuticals in aqueous environmental samples. *Trac-Trend Anal. Chem.* 20, 419-434.

Ternes, T.A., Hirsch, R., Mueller, J., Haberer, K., **1998**. Methods for the determination of neutral drugs as well as betablockers and beta(2)-sympathomimetics in aqueous matrices using GC/MS and LC/MS/MS. *Fresen. J. Anal. Chem.* 362, 329-340.

Ternes, T.A., Janex-Habibi, M-L., Knacker, T., Kreuzinger, N., Siegrist, H., **2005**. *Assessment of Technologies for the Removal of Pharmaceuticals and Personal Care Products in Sewage and Drinking Water Facilities to Improve the Indirect Potable Water Reuse* - EU-Project POSEIDON.

Terzic, S., Senta, I., Ahel, M., Gros, M., Petrovic, M., Barcelo, D., Muller, J., Knepper, T., Marti, I., Ventura, F., Jovancic, P., Jabucar, D., **2008**. Occurrence and fate of emerging wastewater contaminants in Western Balkan Region. *Sci. Total Environ.* 399, 66-77.

Thomas, K.V., Hilton, M.J., **2004**. The occurrence of selected human pharmaceutical compounds in UK estuaries. *Mar. Pollut. Bull.* 49, 436-444.

Togola, A., Budzinski, H., **2007**. Analytical development for analysis of pharmaceuticals in water samples by SPE and GC-MS. *Anal. Bioanal. Chem*. 388, 627-635.

Togola, A., Budzinski, H., **2008**. Multi-residue analysis of pharmaceutical compounds in aqueous samples. *J. Chromatogr. A* 1177, 150-158.

Unceta, N., Gomez-Caballero, A., Sanchez, A., Millan, S., Sampedro, M.C., Goicolea, M.A., Salles, J., Barrio, R.J., **2008**. Simultaneous determination of citalopram, fluoxetine and their main metabolites in human urine samples by solid-phase microextraction coupled with high-performance liquid chromatography. *J. Pharm. Biomed. Anal.* 46, 763-770.

Valentini, F., Compagnone, D., Gentili, A., Palleschi, G., **2002**. An electrochemical ELISA procedure for the screening of 17β - estradiol in urban waste waters. *Analyst* 127, 1333-1337.

van den Brandhof, E.-J., Montforts, M., **2010**. Fish embryo toxicity of carbamazepine, diclofenac and metoprolol. *Ecotox. Environ. Safe.* 73, 1862-1866.

van der Ven, K., Keil, D., Moens, L.N., Hummelen, P.V., van Remortel, P., Maras, M., De Coen, W., **2006**. Effects of the antidepressant mianserin in zebrafish: molecular markers of endocrine disruption. *Chemosphere* 65, 1836-1845.

van der Ven, K., Van Dongen, W., Maes, B.U., Esmans, E.L., Blust, R., De Coen, W.M., **2004**. Determination of diazepam in aquatic samples by capillary liquid chromatography-electrospray tandem mass spectrometry. *Chemosphere* 57, 967-973.

Vasskog, T., Berger, U., Samuelsen, P.-J., Kallenborn, R., Jensen, E., **2006**. Selective serotonin reuptake inhibitors in sewage influents and effluents from Tromsø, Norway. *J. Chromatogr. A* 1115, 187-195.

Vernouillet, G., Eullaffroy, P., Lajeunesse, A., Blaise, C., Gagné, F., Juneau, P., **2010**. Toxic effects and bioaccumulation of carbamazepine evaluated by biomarkers measured in organisms of different trophic levels. *Chemosphere* 80, 1062-1068.

Wells, M.J.M., **2006**. Log D_{OW}: Key to understanding and regulating wastewater-derived contaminants. *Environ. Chem.* 3, 439-449.

Westerhoff, P., Yoon, Y., Snyder, S., Wert, E., **2005**. Fate of endocrine-disruptor, pharmaceutical, and personal care product chemicals during simulated drinking water treatment processes. *Environ. Sci. Technol.* 39, 6649-6663.

Wille, S.M.R., Maudens, K.E., Van Peteghem, C.H., Lambert, W.E.E., **2005**. Development of a solid phase extraction for 13 new generation antidepressants and their active metabolites for gas chromatographic-mass spectrometric analysis. *J. Chromatogr. A* 1098, 19-29.

Xia, K., Bhandari, A., Das, K., Pillar, G., **2005**. Occurrence and fate of pharmaceuticals and personal care products (PPCPs) in biosolids. Conference on Sustainable Land Application. American Society Agronomy, Orlando, FL, pp. 91-104.

Yamamoto, H., Nakamura, Y., Moriguchi, S., Nakamura, Y., Honda, Y., Tamura, I., Hirata, Y., Hayashi, A., Sekizawa, J., **2009**. Persistence and partitioning of eight selected pharmaceuticals in the aquatic environment: Laboratory photolysis, biodegradation, and sorption experiments. *Water Res.* 43, 351-362.

Yu, L., Fink, G., Wintgens, T., Melin, T., Ternes, T.A., **2009**. Sorption behavior of potential organic wastewater indicators with soils. *Water Res.* 43, 951-960.

Zhang, Y., Geißen, S.-U., Gal, C., **2008**. Carbamazepine and diclofenac: removal in wastewater treatment plants and occurrence in water bodies. *Chemosphere* 73, 1151-1161.

Zhang, Z.L., Zhou, J.L., **2007**. Simultaneous determination of various pharmaceutical compounds in water by solid-phase extraction-liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A* 1154, 205-213.

Zuccato, E., Calamari, D., Natangelo, M., Fanelli, R., **2000**. Presence of therapeutic drugs in the environment. *Lancet* 355, 1789-1790.

CHAPTER 3

Direct photodegradation of the anti-epileptic carbamazepine

Carbamazepine, a widely consumed anti-epileptic pharmaceutical, is one of the most commonly detected pharmaceuticals in the environment and has been repeatedly proposed as an adequate marker of anthropogenic pollution. To better assess the environmental persistence of carbamazepine in aqueous matrices, the effect of pH and dissolved oxygen on its direct photodegradation rate was evaluated, using simulated solar irradiation. The photodegradation kinetics were followed by micellar electrokinetic chromatography and a total of seven photodegradation products were identified by mass spectrometry. The results indicate that acidic pH, combined with the absence of dissolved oxygen, results in very high direct photodegradation rates. On the other hand, at basic pH, dissolved oxygen does not interfere with the process and very low rates were observed. At environmentally relevant conditions, carbamazepine has shown to persist to direct photolysis with half-life times between 4.5 and 25 summer sunny days.

CONTENTS

3.1 CONTEXTUALIZATION

Carbamazepine is an anti-epileptic pharmaceutical commonly detected in the environment. As concluded in chapter 2, carbamazepine has been proposed by several authors as a possible marker of anthropogenic pollution, due to its widespread occurrence and high persistence in aquatic environments (Clara et al., 2004; Yu et al., 2009). A correct evaluation of the adequacy of carbamazepine as an anthropogenic pollution marker implies a comprehensive study of its environmental fate. As its solid-water distribution coefficient is low, carbamazepine is expected to be present in the aqueous phase in significant amounts (Ternes et al., 2004). Thus, taking into account its preference to remain in the aqueous phase, photodegradation might be the most relevant process which determines the persistence of this compound in surface waters, particularly in those exposed to sunlight (Boreen et al., 2003; Arnold and McNeill, 2007). In this context, the work here presented intends to assess the relevance of direct photodegradation processes in the environmental persistence of carbamazepine, and to study the effect of relevant environmental factors (such as pH and dissolved oxygen) on the photodegradation rates and on the emergence of photoproducts. In order to follow the photodegradation of carbamazepine and the formation of photodegradation products, a new micellar electrokinetic chromatography (MEKC) method was developed; also, several photodegradation products were identified by mass spectrometry. Some brief introductory considerations about the main topics and concepts involved in this work are presented below.

3.1.1 Relevance of photodegradation processes in the environment

Once in the environment, the fate of organic pollutants is, in general, potentially affected by the same degradation and transportation processes which are more or less relevant according to the compounds' specific characteristics (Petrovic and Barceló, 2007; Glassmeyer et al., 2008). However, pharmaceuticals differ from other contaminants in a particular point: the occurrence of pharmaceuticals in the environment implies that, in most cases, they have passed completely or partially unaffected through the digestive system of humans or animals and through WWTPs biological treatments. This means that they are resistant to acid and enzyme-promoted biodegradation and thus, biodegradation is not expected to be the main process in the elimination of these compounds from the environment; consequently, photodegradation might play a key role on their environmental persistence (Petrovic and Barceló, 2007; Kummerer, 2008). The importance of photochemical processes is, obviously, dependent on the sensitivity of a compound towards light and on the exposure to light (for instance, pharmaceuticals adsorbed onto soils or sediments should not be equally affected by photodegradation due to the lack of light penetration in those matrices

(Kummerer, 2008)). Thus, photodegradation should be considered particularly relevant in surface waters and WWTP which are considerably exposed to solar light irradiation. Concerning the sensitivity of these compounds towards light, they are reasonably expected to undergo photodegradation as they generally possess aromatic moieties and heteroatoms (Boreen et al., 2003). However, there are other factors of extreme importance which dictate the occurrence and extension of photochemical processes, such as the amount of solar light reaching the pollutant, spatio-temporal distribution of the solar light and atmospheric optical properties (Zafiriou et al., 1984).

When exposed to solar radiation, pharmaceuticals can undergo direct or indirect photolysis. Direct photolysis occurs when a compound absorbs a photon which is then able to induce a chemical transformation (Schwarzenbach et al., 2003; Boule et al., 2005). The induction of a chemical change as a direct consequence of absorbing a photon is conceptually the simplest type of photochemical reaction (Zafiriou et al., 1984). On the other hand, indirect photolysis can play an important role on the degradation of pollutants that poorly absorb solar radiation or that resist to direct photolysis and occurs when the phototransformation is indirectly caused by excitation of chromophores present in natural waters (Zafiriou et al., 1984; Boule et al., 2005) (more details about indirect photolysis in the environment will be given in chapter 4).

The presence of oxygen and the pH value are two main factors affecting photodegradation processes in aquatic environments. Oxygen leads to the generation of reactive oxygen species (such as singlet oxygen, superoxide, hydrogen peroxide, hydroxyl radicals) which usually play a major role on mediating photochemical transformations. Moreover, as pharmaceuticals commonly have at least one pH sensitive functional group, pH affects the speciation of ionized and non-ionized forms, which might have distinct photodegradation rates (Boreen et al., 2003). In fact, the main factors affecting direct photodegradation rates (light absorption rate and quantum efficiency of the process - see section 3.1.3) are pH dependent (Arnold and McNeill, 2007). The effect of pH and dissolved oxygen in the direct photodegradation rate of carbamazepine and its environmental repercussions will be discussed along this chapter.

3.1.2 Outdoor exposure *versus* simulated photodegradation studies

Nowadays, photodegradation studies are often performed using solar light simulators, most commonly equipped with arc xenon lamps and appropriate filters (Vione et al., 2006; Guerard et al., 2009; Sevilla-Morán et al., 2010). Simulating environmental conditions is always a huge challenge due to the high number and complexity of the involved variables. The simulation of

sunlight outdoor exposure is not an exception; yet, the numerous advantages offered by solar simulators should also be taken into account when one has to choose between the two possibilities. A summary of the main factors to consider when comparing sunlight outdoor exposure and the use of solar simulators is presented in Table 3.1. One of the main problems involving simulated solar light sources is the difficulty in reproducing the solar spectrum. In Figure 3.1, the solar spectrum and a 1500 W arc xenon lamp spectrum (lamp used in the photodegradation tests here described) are presented. In this context, the most relevant difference is the lower spectral irradiance of the arc xenon lamp, particularly between 290 and 305 nm, which is the most significant wavelength interval for the majority of pharmaceuticals (in terms of sensitivity towards light). On the other hand, solar light simulators offer predictability, repeatability, steady output and the possibility of carrying out tests for uninterrupted time. Ultimately, real environmental conditions, in what concerns solar irradiation, can only be offered by outdoor exposure of samples. However, the performance of solar simulators is often considered to be satisfactory. A proper conversion of kinetic parameters obtained by using solar simulators into real outdoor values is a key point in producing results with environmental significance when using these devices.

Table 3.1. Comparison between solar light outdoor exposure and the use of solar simulators to perform photodegradation tests.

Outdoor exposure – solar light	Solar simulators
Exposure to the real solar spectrum	Difficulty in properly simulating the entire range of the solar light spectrum
Limited availability of solar radiation: day/night cycle	Possibility of carrying out tests 24 h/day
Diurnal and seasonal variations of global solar irradiance	Steady output and control over the total irradiance
Unpredictable weather conditions	Predicable conditions; possible control over temperature, humidity, etc
Impossible to repeat the same test under the exact same conditions	Repeatable conditions
Real outdoor results are directly obtained	Obtained results need to be properly converted into environmentally relevant data

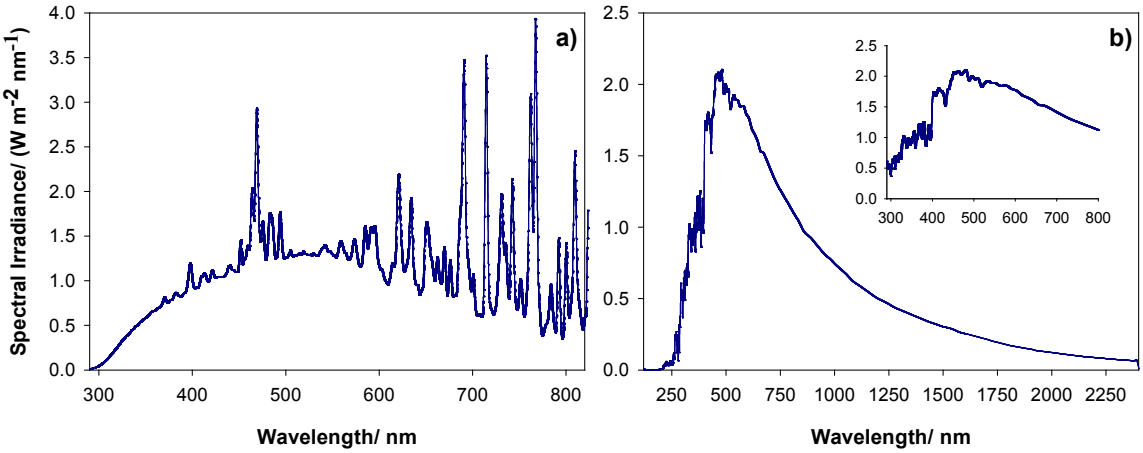

Figure 3.1. a) Spectral irradiance of a 1500 W arc xenon lamp when using an outdoor UV filter, as given by the manufacturer (Solarbox 1500, Co.fo.me.gra, Italy). The spectrum is referred to a total irradiance of 550 W m^{-2} between 290-800 nm. b) Solar spectral irradiance obtained under the SORCE project (NASA, 2008). The spectrum is referred to 23rd July 2008; the values were averaged in order to provide daily solar irradiance.

3.1.3 Quantum yield determination

The quantum yield (ϕ) of a photolysis process can be defined as the number of moles of consumed reactant for each mole of photons absorbed by the same reactant. Alternatively, it can also be described as the ratio between the photodegradation rate and the rate of light absorption (Boule et al., 2005; Braslavsky, 2007). An important detail about this definition should be emphasized: it does not distinguish between the quantum efficiency of the primary photochemical processes and subsequent secondary reactions. To avoid misconceptions, the term overall quantum yield is commonly used and is referred to the yield of removal of a specific reactant. Quantum yield values are, in general, between 0 and 1; values higher than 1 strongly suggest the occurrence of secondary reactions (Boule et al., 2005). However, quantum yields between 0.0001 and 0.1 are usually found for pharmaceuticals with environmental photodegradation half-lives between several days and minutes (Arnold and McNeill, 2007). When considering the environmental relevance of photodegradation processes, this parameter is of crucial importance to assess the persistence of a contaminant and its determination also allows a valid comparison between other studies reported in the literature.

The determination of photodegradation quantum yields requires a method to measure the photon flux emitted by the light source. The two most common approaches found in literature are

chemical actinometry and radiometry. Chemical actinometers are chemical systems that suffer a photochemical reaction for which the quantum yield is accurately known. By subjecting the actinometer to the same irradiation conditions of the samples, it is possible to determine the intensity of the incident light through the determination of its photodegradation kinetics (Kuhn et al., 2004; OECD, 2008). The other approach consists on the direct measurement of irradiance using a calibrated radiometer. In this work, the determination of the quantum yield was performed by radiometry, following the approach detailed in the subsequent paragraphs, adapted from Chiron et al. (2006) and Neamtu and Frimmel (2006).

Considering *rate* λ_i the photodegradation rate of a compound induced by the absorption of radiation with wavelength λ_i and $I_{\lambda i}^{abs}$ the rate of light absorption at λ_i, the photolysis quantum yield is given by equation 3.1:

$$\Phi_{\lambda i} = \frac{\text{rate } \lambda i}{I_{\lambda i}^{abs}} \quad (3.1)$$

Moreover, $I_{\lambda i}^{abs}$, the rate of light absorption at λ_i, can be determined according to equation 3.2:

$$I_{\lambda i}^{abs} = I_{\lambda i}^{0} \times \left(1 - 10^{-\varepsilon_{\lambda i} \times b \times C_0}\right) \quad (3.2)$$

where C_0 is the initial concentration of the compound in solution (mol L^{-1}), $I_{\lambda i}^{0}$ is the lamp emission intensity at the wavelength λ_i (Ein L^{-1} s^{-1}), ε is the molar absorptivity of the compound at λ_i (L mol^{-1} cm^{-1}) and b is the path length inside the photoreactor (cm). $I_{\lambda i}^{0}$ can be directly determined by converting the spectral irradiance of the lamp (usually given in W m^{-2} nm^{-1}) into number of photons emitted per unit of sample volume per unit of time (Ein L^{-1} s^{-1}). One Einstein (Ein) corresponds to one mole of photons. The use of this unit is considered obsolete and is not recommended by IUPAC; however, it is still universally used within this context.

According to equations 3.1 and 3.2, the photolysis quantum yield at λ_i is given by equation 3.3:

$$\Phi_{\lambda i} = \frac{C_0 \times k_i}{I_{\lambda i}^{0} \times \left(1 - 10^{-\varepsilon_{\lambda i} \times b \times C_0}\right)} \quad (3.3)$$

with k_i corresponding to the first order rate constant (s^{-1}) at λ_i. However, taking into account the experimental impossibility of determining the rate constant of a photodegradation process corresponding to the absorption of a single wavelength (when the light source is not

monochromatic), the photodegradation quantum yield is often determined considering an overall average over the lamp emission wavelength range as given by equation 3.4:

$$\phi_{ave} = \frac{C_0 \times k}{\sum(I^0_{\lambda i} \times (1 - 10^{-\varepsilon_{\lambda i} \times b \times C_0}))} \quad (3.4)$$

where *k* is the apparent first order rate constant (s^{-1}). According to these equations, the units of the average quantum yield should be mol Ein^{-1}. Taking into account that one Ein is equivalent to one mole of photons, the quantum yield is a dimensionless quantity.

3.1.4 Basic concepts of capillary electrophoresis

The main principle of electrophoresis is the separation of charged molecules based on their movement through a fluid when an external constant electric field is applied (Weinberger, 2000; Marina et al., 2005). Capillary electrophoresis is one of the several possible formats of electrophoretic techniques and consists on the usage of a thin capillary as the physical support for the electrophoretic separation. Instrumental requirements to conduct capillary electrophoresis separations are relatively simple. In fact, in the early beginning of this technique, the work was majorly performed using homemade equipments. Nowadays, basic instrumental configuration mainly consists on (Marina et al., 2005):

- High voltage power supply;
- Reservoirs containing electrolytic solutions and samples, often incorporated in auto sampler devices;
- Two electrodes at the inlet and outlet, generally made of platinum;
- Capillary, usually inside a thermostated compartment;
- Detection system;
- Software to control the instrument and manage data acquisition and processing.

Overall, capillary electrophoresis offers several advantages when compared to chromatographic techniques such as HPLC. It requires much smaller quantities of reagents and only a few nanoliters of sample. Moreover, capillaries are less expensive than HPLC columns and are much more versatile (the surface of the capillary columns, which is determinant in the separation process, can be easily modified by manipulating the pH, using capillary coatings or even (pseudo)stationary phases). Capillary electrophoresis also allows the separation of molecules

within a wide range of molecular weights and charges (Weinberger, 2000). In general, it is characterized by better efficiency and separation resolution than HPLC (Marina et al., 2005).

Briefly, electrophoretic separations are driven by two major components: electrophoretic mobility and electroosmotic flow (EOF) (Marina et al., 2005). The electrophoretic mobility (μ_{ep}) of charged species in an electrolytic solution is a consequence of the electrostatic force exerted when a constant electric field is applied. At constant velocity, the electrophoretic mobility ($cm^2\ V^{-1}\ s^{-1}$) can be expressed as:

$$\mu_{ep} = \frac{\upsilon_{ep}}{E} \quad (3.5)$$

where E ($V\ cm^{-1}$) is the electric field strength and υ_{ep} ($cm\ s^{-1}$) is the migration velocity of the ionic species. The electrophoretic mobility depends on the charge and size ratio (q/r) and on the viscosity (η) of the electrolytic solution (and therefore on temperature and nature of the electrolytic solution), according to the following equation:

$$\mu_{ep} = \frac{q}{6\pi\eta r} \quad (3.6)$$

The EOF is the liquid flow originated by the presence of an electric field, when an electrolytic solution is in contact with the charged inner capillary wall (Marina et al., 2005). Before the first use, capillaries are usually conditioned, consisting on the ionization of the silanol groups of its inner wall. As a consequence, the capillary surface remains negatively charged and a high number of counterions, placed adjacently to the capillary wall, originate an electric double layer. This double layer is constituted by ions that are strongly bound and immobilized and by a second mobile cationic layer that, under an electric field, migrates towards the cathode, mobilizing the electrolytic solution inside the capillary and resulting on the EOF (Skoog et al., 1998; Weinberger, 2000; Marina et al., 2005). The EOF depends on the zeta potential (ζ, potential across the double layer), the dielectric constant of the solution (ε) and on the viscosity (η) of the electrolytic solution according to:

$$\upsilon_{eo} = \frac{\varepsilon E \zeta}{4\pi\eta} \quad (3.7)$$

As a consequence of equation 3.7, there is a large number of experimental variables that might be manipulated to modify the EOF, such as pH, nature and concentration of the electrolytic solution, addition of organic modifiers, temperature or composition of the capillary inner wall surface (Weinberger, 2000; Marina et al., 2005). The EOF is a key concept in capillary electrophoresis as it has the ability to modify the migration velocity of species depending on whether they are moving

in the same or in the opposite direction of the EOF. In fact, the real electrophoretic mobility of charged species is a function of the EOF, according to the following equation:

$$\mu_{ep} = \mu_a \pm \mu_{eo} \quad (3.8)$$

where μ_a and μ_{eo} are the apparent (experimentally measurable) electrophoretic mobility of ionic species and of the EOF, respectively (Skoog et al., 1998; Weinberger, 2000; Marina et al., 2005). The control and reproducibility of the EOF is absolutely critical to capillary electrophoresis as it can control the whole separation process. It is also the flat profile of the EOF across the capillary diameter which enables the high resolution and peak efficiency which characterizes capillary electrophoresis (Weinberger, 2000).

Capillary electrophoresis appears in a variety of separation modes. The simplest separation mode, capillary zone electrophoresis (CZE), consists on the usage of a capillary filled with an electrolytic solution (also commonly designated as running buffer) and the separation of charged molecules is based on differences on their electrophoretic mobility (Marina et al., 2005). Obviously, this separation mode is limited to charged molecules, which considerably restricts the applicability of this technique: neutral molecules migrate through the capillary at the same velocity of the EOF, without any separation (Marina et al., 2005). The expansion of capillary electrophoresis to the separation of neutral molecules was possible by the development of electrokinetic capillary chromatography (EKC) (Terabe et al., 1984; Terabe et al., 1985). Ultimately, this separation mode is a combination of the concepts involved both in CZE and chromatography and consists on the introduction of a pseudostationary phase into the running buffer. Electrophoretic mobility and EOF continue to be the driving forces responsible for the movement of the pseudostationary phase and solutes through the capillary. However, in this case, the separation of neutral molecules is dictated by their partition between the pseudostationary and the aqueous phases (Marina et al., 2005). The pseudostationary phase might consist on a large variety of additives in the running buffer. Most commonly, a surfactant is used and, in this case, the micelles (aggregates of surfactant molecules spontaneously formed in aqueous solution) are the pseudostationary phase. This separation mode is known as micellar electrokinetic chromatography (MEKC) and separation is based on the partition of the analytes between the micelle and the aqueous phase (Weinberger, 2000). Sodium dodecylsulphate (SDS) is often used as surfactant agent due to its high water solubility, degree of lipid solubilizing power and also due to its low critical micellar concentration (8 mM) which are the main criteria to take into account for the selection of an adequate surfactant (Weinberger, 2000). When using SDS, negatively charged micelles are formed which will, consequently, migrate towards the anode. Thus, the presence of the

EOF is of crucial importance to enable the movement of the micelles towards the detector at the cathode end (Pranaityté and Padarauskas, 2006).

MEKC methods are widely used in the analysis of anti-epileptic pharmaceuticals and their metabolites in biological samples (Smyth and McClean, 1998; Thormann et al., 2001; Pucci and Raggi, 2005). In this work, it is described the application of a MEKC methodology to the separation of the anti-epileptic carbamazepine from its photodegradation products.

3.2 MATERIALS AND METHODS

3.2.1 Chemicals

All chemicals used were of analytical grade: carbamazepine (99%, Sigma), sodium dodecylsulphate (99%, for electrophoresis, Sigma-Aldrich), hexadimethrine bromide (polybrene, Sigma-Aldrich), sodium chloride, ethylvanillin (99%, Aldrich), sodium tetraborate (borax, Riedel-de Haën), sodium hydroxide (Fluka), formic acid (98%, Fluka) and acetonitrile (HPLC gradient grade, VWR, Prolabo).

Ultra-pure water, used in the preparation of standard solutions, running buffer and irradiation samples, was obtained using a Milli-Q Millipore system (Milli-Q plus 185).

3.2.2 Irradiation experiments

3.2.2.1 Irradiation apparatus

The irradiation experiments were performed with the Solarbox 1500 (Co.fo.me.gra, Italy) equipped with a 1500 W arc xenon lamp and special outdoor UV filters that restrict the transmission of light with wavelengths below 290 nm. The uniformity of the irradiation was provided by a parabolic reflection chamber, whereas the temperature of the irradiation chamber was maintained by an air cooled system. During the irradiation, the irradiance was kept constant at 550 W m^{-2} (290 - 800 nm); a multimeter (Co.fo.me.gra, Italy) equipped with a black standard temperature sensor and a UV 290 - 400 nm large band sensor was used to monitor the irradiance levels and temperature. A schematic representation of the irradiation chamber is shown in Figure 3.2.

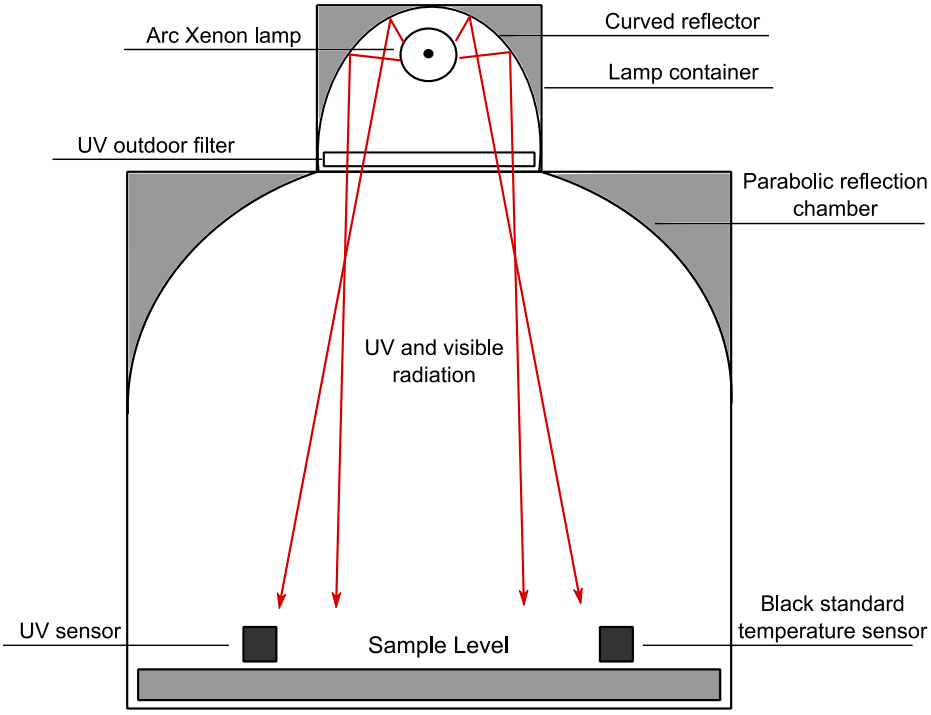

Figure 3.2. Scheme representing a lateral cross-section of the solar light simulator used in the irradiation experiments (Solarbox 1500, Co.fo.me.gra, Italy).

3.2.2.2 Sample preparation and sampling

Carbamazepine stock solutions were prepared with ultra-pure water with a final concentration of 9.5 mg L^{-1} (approximately half of its solubility in water) and were stored in dark glass bottles at 4 ºC, for no more than 1 month.

Stock solutions were irradiated in triplicate using 25 mL quartz tubes with a diameter of 1.5 cm. Each set of experiments was accompanied by dark controls inside the irradiation chamber, also in triplicate, foiled several times with aluminum paper. The quartz tubes were suspended inside the chamber using a homemade metallic holder which guaranteed that samples were homogeneously irradiated. Irradiation experiments were carried out up to 24 h. Sampling consisted on the collection of 2 mL aliquots of all the irradiated samples at specific time intervals. The aliquots were stored at 4 ºC and analyzed by MEKC within the next 3 days.

3.2.2.3 Study of the effect of pH and dissolved oxygen

To study the photodegradation behavior of carbamazepine under different conditions, experiments were conducted at four different pH: 2.9, 4.0, 5.8 and 9.0. The pH of the stock solutions was adjusted using 1 M sodium hydroxide or formic acid prior to irradiation. Also, the effect of the dissolved oxygen in the aqueous solutions was evaluated. Accordingly, each experiment was repeated after sparging the solutions with nitrogen or oxygen in order to remove or saturate the solutions with oxygen, respectively. The samples were sparged with the referred gases during approximately 1 min per mL of solution. During the sampling procedure, the quartz tubes were kept under oxygen or nitrogen atmosphere depending on the sample under study.

3.2.3 Capillary electrophoresis

To follow the degradation of carbamazepine and the appearance of its photodegradation products, a MEKC based methodology was developed. The capillary electrophoresis analyses were performed using a commercial instrument (Beckman P/ACE MDQ (Fullerton, CA, USA)), equipped with a photodiode array UV-Vis detection system.

3.2.3.1 Capillary column conditioning and coating

A fused-silica capillary with a total length of 50 cm (40 cm to detector) and 75 μm of internal diameter was used. Approximately 1 mm of the capillary external coating was removed by burning the extremities prior to conditioning; the capillary extremities were then polished to increase the efficiency of the method and decrease the baseline noise. The new capillaries were first conditioned with 1 M NaOH for 30 min followed by ultra-pure water for 15 min. Subsequently, to proceed with the capillary coating, it was flushed with hexadimethrine bromide (polybrene) 0.5% (*w/v*) in 0.5 M NaCl for 20 min, as described by Pranaityté and Padarauskas (2006). Then, the capillary was washed with ultra-pure water for 2 min, followed by a 20 min flushing with running buffer. All the capillary conditioning and coating steps were performed at a pressure of 20 psi.

The capillary was washed with running buffer for 20 min at the beginning of each working day and with ultra-pure water for 5 min at the end of the day. When not in use the capillary extremities were left immersed in ultra-pure water to ensure the stability of the capillary coating.

3.2.3.2 Standard solutions and running buffer

Ethylvanillin was used as internal standard (IS) in the MEKC analysis. IS stock solution was prepared by dissolving ethylvanillin in acetonitrile (approximately 10% of the total volume) and further diluting it with ultra-pure water to a final concentration of 167 mg L^{-1}. The solution was stored at 4 ºC under N_2 atmosphere.

For the MEKC calibration curve, six standard solutions, with concentrations ranging from 0.5 to 8 mg L^{-1}, were prepared by diluting the carbamazepine stock solution. Standard solutions also contain IS (final concentration of 3.34 mg L^{-1}), SDS and borax with the same concentrations of the running buffer. Standard solutions were analyzed in quadruplicate.

The running buffer consisted on 25 mM borax and 50 mM SDS, pH 9.2, freshly prepared every 2 days and stored at 4º C.

All the solutions were filtered through a membrane filter (pore size 0.22 μm, Millex-GV) before analysis.

3.2.3.3 Sample preparation prior to MEKC analysis

MEKC samples were prepared by adding SDS, borax and IS to the irradiated samples in order to obtain a final concentration of 50 mM, 25 mM and 3.34 mg L^{-1}, respectively.

3.2.3.4 Separation conditions

Samples were injected for 4 s at 0.5 psi. Electrophoretic separations were performed in direct polarity with a positive potential supply of 20 kV for 13 min. The temperature of the capillary was maintained at 25 ºC. The resulting current was approximately 110 μA. Detection of carbamazepine and carbamazepine photodegradation products were monitored at 210 nm. Running buffer vials were changed every 3 runs.

3.2.4 Identification of photodegradation products by mass spectrometry

The identification of carbamazepine photodegradation products was performed by electrospray mass spectrometry (ESI-MS), using N_2-sparged samples collected after 30 min of irradiation at pH 2.9, and not sparged samples collected after 24 h of irradiation at pH 5.8. Positive-ion ESI-MS and ESI-MS^2 were carried out on a Micromass (Manchester, UK) Q-TOF2 hybrid tandem mass spectrometer. For ESI analysis, irradiated samples were diluted in methanol (0.1% formic acid *v/v*). Samples were introduced at a flow rate of 10 μL min^{-1} into the ESI source. In the MS and MS^2 experiments, the time-of-flight (TOF) mass resolution was set to approximately 9000. The cone voltage was 35 V, and the capillary voltage was 3 kV. The source temperature was 80 ºC and the desolvation temperature was 150 ºC. MS^2 spectra were obtained using argon as the collision gas with the collision energy set between 18 and 40 eV. In order to calibrate the MS spectra, the lock mass was the calculated monoisotopic mass/charge of the carbamazepine ion $[M+H]^+$. The data was processed using MassLynx software (version 4.0).

3.3 RESULTS AND DISCUSSION

3.3.1 Optimization of the MEKC method

The performance of the adopted methodology was evaluated by comparing the results obtained using uncoated and coated capillary columns.

Initially, irradiated samples were analyzed with an uncoated capillary after being conditioned with 1 M NaOH for 30 min followed by ultra-pure water for 10 min. In Table 3.2, results of repeated injections of a carbamazepine standard solution (mean peak area, migration time and relative standard deviation) are displayed. The results show that the method provides good repeatability of the evaluated parameters when using standard solutions. However, resolution of the peaks due to carbamazepine and to its photodegradation products was unsatisfactory in the samples irradiated for long periods of time. Also, when analyzing these samples, the repeatability of peak area was strongly affected, showing the lack of efficiency of the methodology under these conditions. Taking into account the obtained results, a dynamically coated capillary was used. Dynamically coated capillaries have the advantage of improving the reproducibility (Vanhoenacker et al., 2004; Pranaityté and Padarauskas, 2006) by decreasing the interaction between the analytes and the capillary inner wall, avoiding variations due to possible modifications in the chemical

structure of the bare silica capillary surface between repeated injections. Moreover, this type of coating has already been reported as a promising tool to improve the separation efficiency (Erny et al., 2009). This procedure is easy to implement and, on the whole, consists on coating the capillary surface with a buffer containing a multiple charged polycation (polybren) and then flushing the positively charged surface with an anionic surfactant (SDS). The first layer of SDS interacts with the cationic surface of polybren and a second layer of SDS is formed by establishing hydrophobic interactions with the first one, resulting in a highly negatively charged surface. The chemical modifications of the capillary surface during the procedure are illustrated in Figure 3.3.

Table 3.2. Comparison of the MEKC methodology repeatability considering the usage of a coated and an uncoated capillary: mean and relative standard deviations (R.S.D.) of the ratio between carbamazepine (CBZ) and internal standard (IS) peak areas and CBZ migration time (n = 4).

Composition of the standard solution	CBZ /IS peak areas		CBZ migration time	
	Mean (a. u.)	*R.S.D. %*	*Mean (min)*	*R.S.D. %*
Uncoated Capillary				
CBZ stock + IS	9.36	1.61	10.67	0.04
Coated Capillary				
CBZ stock + IS	9.29	4.08	10.36	0.10
50% CBZ stock + 50% running buffer + IS	4.26	0.44	9.95	0.25
90% CBZ stock + 10% running buffer + IS	7.13	0.78	10.29	0.35

After coating, the performance of the new methodology was evaluated (results presented in Table 3.2). Similarly to the previous case, the method proved to have good repeatability, however, in this case, it was possible to separate carbamazepine from its photo-irradiation products. The comparison of the electrochromatograms of a sample irradiated for 24 h, obtained with a coated capillary (A) and an uncoated capillary (B) is shown in Figure 3.4. The hypothesis of co-migration of carbamazepine and photodegradation products in the latter case, as well as the ability of the coated capillary to perform the separation efficiently, is clearly demonstrated. Consequently, all the subsequent analyses were performed using the capillary coating here described.

Furthermore, the addition of running buffer to the irradiated samples, prior to MEKC analysis (as referred in the experimental section), substantially improved the repeatability of the ratio between carbamazepine and IS peak areas. The addition of 10% of the running buffer was chosen (instead of 50%) to avoid a substantial dilution of the samples.

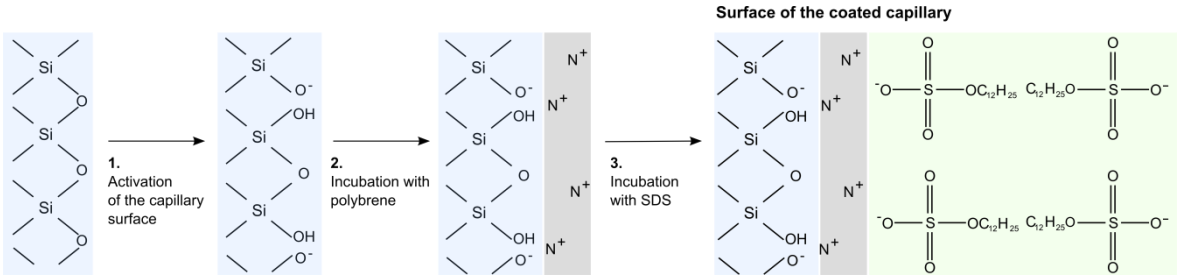

Figure 3.3. Schematic representation of the capillary coating used in the developed methodology.

The method turned out to be repeatable between successive runs without the need to recoat the capillary. It was also possible to use an efficient and faster washing procedure between runs (2 min) when compared to traditional MEKC methodologies, increasing even more the speed of analysis that characterize capillary electrophoresis methods. It was also verified the good stability of the coating for several weeks, for which it is indispensable the storage of the capillary filled with water and with its extremities immersed in ultra-pure water.

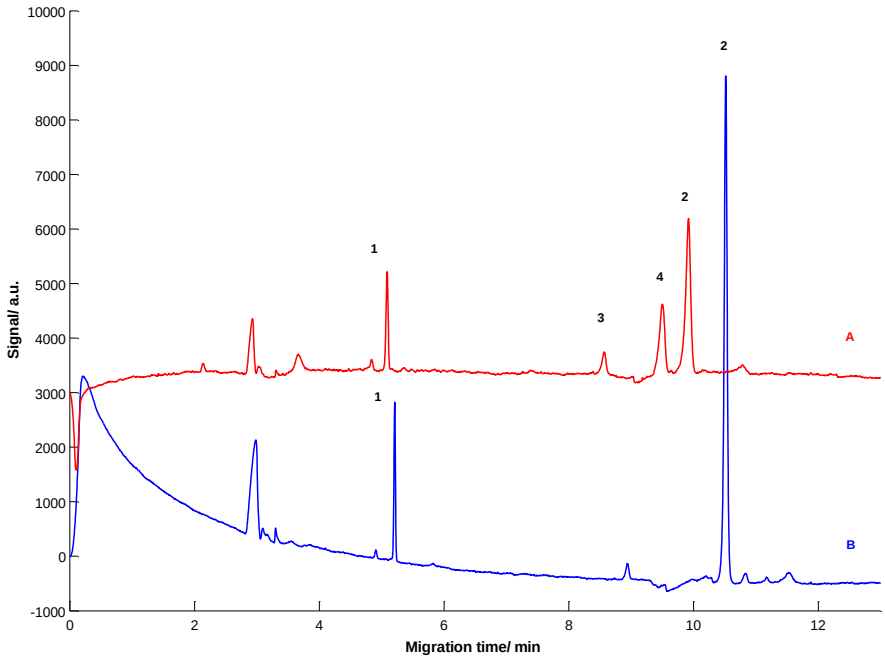

Figure 3.4. Electrochromatograms of samples resulting from the irradiation of carbamazepine for 24 h, obtained using a coated capillary (A) and an uncoated capillary (B). Peak identification: 1- Ethylvaniline (internal standard); 2 – Carbamazepine; 3 and 4 – photodegradation products. Experimental conditions: capillary 0.5 m length (0.4 to detector), 75 µm internal diameter, applied voltage 20 kV, capillary temperature 25 ºC, running buffer 50 mM SDS and 25 mM borax, detection at 210 nm.

3.3.2 Calibration curve for MEKC

A linear calibration curve was obtained, by means of a least-squares linear regression, using six standard solutions with concentrations ranging from 0.5 to 8.0 mg L^{-1}. The linear regression was based on the mean ratio between the peak area of carbamazepine and the peak area of the IS that resulted from four replicate injections of each standard solution as a function of the standard solution concentration. The equation of the regression curve is given by $y = (0.923 \pm 0.002)\ x - (0.075 \pm 0.009)$. The correlation coefficient takes the value of 1.000, confirming the excellent linear response of the adopted methodology in the studied range of concentrations. Additionally, the limit of detection (LOD) and of quantification (LOQ) were determined according to $3s_{x/y}/b$ and $10s_{x/y}/b$, respectively, where b is the slope and $s_{x/y}$ is the residual standard deviation of the determined linear regression (J.N. Miller and Miller, 2005). Accordingly, the LOD and LOQ values are 0.040 and 0.134 mg L^{-1}, respectively.

3.3.3 Carbamazepine photodegradation under simulated solar irradiation

The direct photodegradation rate of carbamazepine was evaluated in aqueous solutions with a concentration of 9.5 mg L^{-1} and pH values of 2.9, 4.0, 5.8 and 9.0. To better understand the role of oxygen and reactive oxygen species in the photodegradation process of this pharmaceutical, carbamazepine solutions were irradiated in an oxygen saturated medium (solutions sparged with O_2 prior to irradiation) and in a nitrogen-deoxygenated medium (solutions sparged with N_2). In Figure 3.5, the percentages of carbamazepine photodegradation as a function of irradiation time for samples not sparged (*a*), sparged with O_2 (*b*) and sparged with N_2 (*c*) are shown. It is clearly evident that higher percentages of photodegradation were attained at pH 2.9 for all the irradiated solutions. Moreover, it is also clear that the photodegradation process strongly depends on the amount of dissolved oxygen present in test solutions. For example, at pH 2.9 the oxygenated solutions reached approximately 85% of degradation after 24 h of irradiation, while in the nitrogen-deoxygenated solution the same value was observed in a period of 1 h. However, this dependence is only observed at low pH; significant differences in the photodegradation extent tend to disappear at high pH values. Very low photodegradation was observed at the highest pH studied (9.0) in all oxygenation levels.

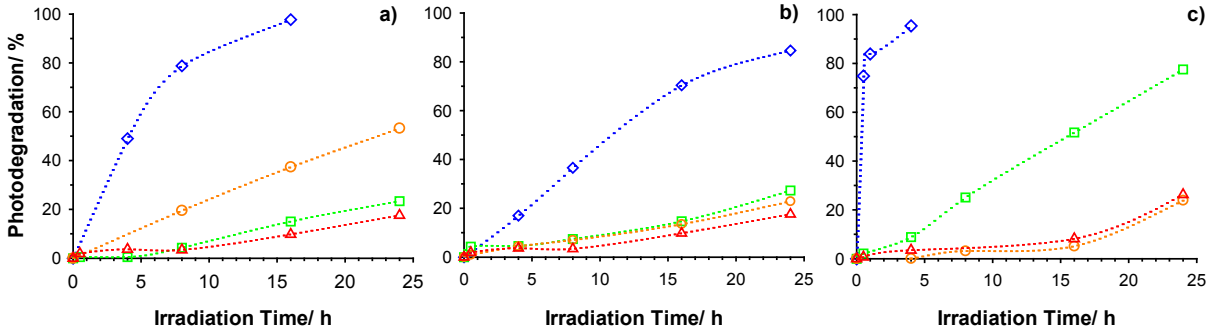

Figure 3.5. Photodegradation of carbamazepine as a function of the irradiation time at different pH: pH 2.9, 4.0, 5.8 and 9.0 represented in blue, green, orange and red, respectively. a) Not sparged; b) sparged with O_2; c) sparged with N_2. Each point corresponds to the mean percentage of photodegradation of three sample replicates. Relative standard deviations are below 10%.

To allow a better comparison of results obtained under distinct experimental conditions, the determination of kinetics parameters was performed by fitting a first order kinetics to each set of results. Considering the Naperian logarithm of the ratio between the concentration of carbamazepine at a give irradiation time (C_t) and the initial concentration of carbamazepine (C_0) as a function of the irradiation time, linear regressions were obtained (according to equation 3.9). Correlation values (r) for solutions sparged with O_2 and not sparged range from 0.97 to 0.99; solutions sparged with N_2 have lower correlation values (between 0.89 and 0.98). The satisfactory linearity of the regressions allowed the determination of the apparent pseudo-first order photodegradation rate constants (k) and half-life times ($t_{1/2}$). The results are presented in Table 3.3.

$$\ln\frac{C_t}{C_0} = -kt \quad (3.9)$$

The most relevant result was obtained for degradation in deoxygenated solutions at pH 2.9 in which the photodegradation rate of carbamazepine was increased by a factor of 6 and 17 when compared with non-sparged and O_2-sparged test solutions, respectively. A considerable increase on the photodegradation rate (by a factor of 6) was also observed at pH 4.0 in nitrogen-deoxygenated solutions. The pH dependence of the photodegradation rates is illustrated in Figure 3.6. The presence of oxygen undoubtedly inhibits the degradation process; the higher the concentration of dissolved oxygen, the lower the photodegradation rate. However, and as it was stated before, the effect of the oxygenation level of the medium is negligible at pH 9.0.

Table 3.3. Correlation coefficient (r), half-life time ($t_{1/2}$) and apparent photodegradation rate constant (k) of carbamazepine obtained by fitting a first order kinetics model to the photodegradation data, considering different pH and dissolved oxygen conditions. n represents the number of points used in the linear regression and σ represents the standard deviation. Half-life times converted to units equivalent to summer sunny days (SSD) and average quantum yields (ϕ_{ave}) of carbamazepine photolysis are also presented (discussed in section 3.3.4).

	pH	r	n	$t_{1/2} \pm \sigma$ /(h)	$k \pm \sigma$ /(h^{-1})	$t_{1/2} \pm \sigma$ /(SSD*)	Φ_{ave}
Not Sparged	2.9	0.994	4	2.9 ± 0.2	0.24 ± 0.02	-	6.4×10^{-5}
	4.0	0.986	5	63 ± 6	0.011 ± 0.001	-	2.9×10^{-6}
	5.8	0.992	7	17 ± 1	0.040 ± 0.002	4.5 ± 0.3	1.1×10^{-5}
	9.0	0.987	5	95 ± 9	0.007 ± 0.001	25 ± 2	2.0×10^{-6}
Sparged with O_2	2.9	0.996	5	8.6 ± 0.5	0.081 ± 0.004	-	2.1×10^{-5}
	4.0	0.971	6	58 ± 7	0.012 ± 0.001	-	3.2×10^{-6}
	5.8	0.975	5	36 ± 5	0.019 ± 0.003	9 ± 1	5.1×10^{-6}
	9.0	0.993	6	67 ± 4	0.010 ± 0.001	18 ± 1	2.7×10^{-6}
Sparged with N_2	2.9	0.925	4	0.5 ± 0.1	1.3 ± 0.3	-	3.5×10^{-4}
	4.0	0.980	6	12 ± 1	0.060 ± 0.006	-	1.6×10^{-5}
	5.8	0.888	5	66 ± 20	0.011 ± 0.003	17 ± 5	2.9×10^{-6}
	9.0	0.924	5	63 ± 15	0.011 ± 0.003	17 ± 4	2.9×10^{-6}

* 1SSD – unit equivalent to 1 summer sunny day (clear sky) at 45ºN latitude. Results are shown only for experiences performed at relevant pH values for surface waters (5.8 and 9.0)

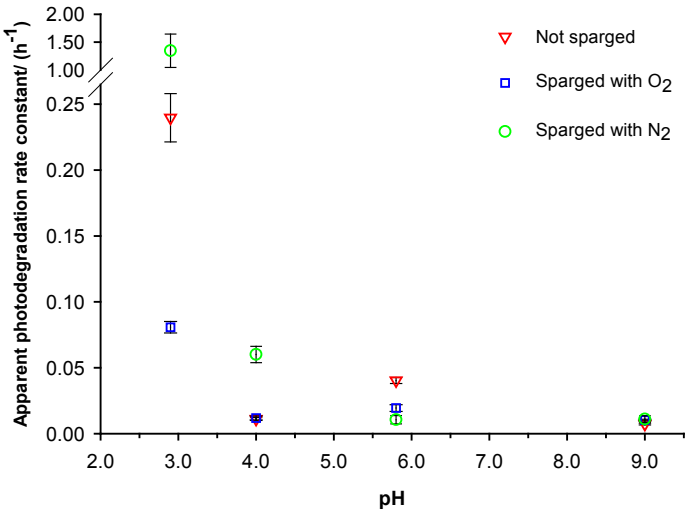

Figure 3.6. pH dependence of the apparent photodegradation rate constant of carbamazepine under different experimental conditions.

The described results point out that reactive oxygen species (such as singlet oxygen) do not have a significant role on the degradation pathway of carbamazepine, seeing that the presence of molecular oxygen did not increase the rate of the degradation process. In addition, and considering the fact that at low pH and in the presence of oxygen, significantly lower degradation rates were observed and that triplet excited states are efficiently quenched by oxygen, it is predictable that one of the most significant photodegradation pathways of carbamazepine arises from its triplet excited states that were quickly deactivated in highly oxygenated solutions. Another tentative hypothesis to explain the observed phenomenon is the possible formation of hydrated electrons. Hydrated electrons, unitary negative charges chemically unbounded to any particular atom, are considered the most elementary and reactive nucleophiles (Anbar and Hart, 1964). Hydrated electrons have been shown to be photoproduced by a wide variety of aromatic compounds, and quickly react with organic compounds that have electronegative atoms (Anbar and Hart, 1964; Joschek and Grossweiner, 1966; Zepp et al., 1987). One known compound that photoproduces hydrated electrons is acridine (Joschek and Grossweiner, 1966; Kellmann and Tfibel, 1980, 1982) which is one of the most common direct photodegradation product of carbamazepine (Chiron et al., 2006) and was identified in this study (see section 3.3.6). The possible production of hydrated electrons during the irradiation experiments, and its reactivity towards carbamazepine, is a possibility that would also explain the degradation decrease in highly oxygenated mediums, taking into account that hydrated electrons are effectively scavenged by molecular oxygen (Zepp et al., 1987).

Another reasonable explanation (but again, tentative) arises from the pH dependence of the results suggesting that different forms of carbamazepine could be implicated. Several cases of pharmaceuticals that have different direct degradation rates which vary with the predominant form in solution (cationic or anionic forms) have been reported (Arnold and McNeill, 2007). It is also reported in the literature that the quantum yield of a degradation process could be pH dependent when different anionic or cationic forms are present (Arnold and McNeill, 2007). In the particular case of carbamazepine, it has a *pKa* of 13.9 (Jones et al., 2002) related to the deprotonation of the NH_2 group and a *pKa* of 2.3 (Nghiem et al., 2005) related to the protonation of the amino groups (carbamazepine structure is shown in Figure 8, section 3.3.6). This means that at environmentally relevant pH, carbamazepine should be present in its neutral form. However, at the tested pH 2.9, a protonated form of carbamazepine exists in equilibrium with the neutral form at a concentration which might be high enough to interfere with the photodegradation rate. Nevertheless, taking into account the observed pH dependence, the existence of a protonated form of carbamazepine at very low pH might not be enough to explain the obtained results, suggesting that a different pH dependent mechanism might be operating. To fully elucidate this point, further research is needed,

including the mechanistic study of the photodegradation processes of carbamazepine at different pH.

3.3.4 Determination of the apparent quantum yield of carbamazepine

The determination of the quantum yield is crucial to allow the comparison of results between different studies. Moreover, the quantum yield is an important parameter to explain differences in photodegradation rates of compounds with similar spectral overlap with the sunlight. Note that compounds with very similar spectral overlap with the sunlight spectrum and with distinct quantum yields should resist to photodegradation processes differently.

The carbamazepine's photodegradation quantum yield was determined considering an overall average over the lamp emission wavelength range (290 – 800 nm) and respective emission intensities, directly extracted from the lamp spectrum (as given by the manufacturer), for the irradiance level used during the experiments (average value: 550 W m^{-2}, 290 – 800 nm). Emission lamp spectrum and absorption spectrum of carbamazepine are shown in Figure 3.1a) (section 3.1.2) and Figure 3.7, respectively. The calculation of the quantum yield was made considering a diameter of the cylindrical photoreactor of 1.5 cm, a volume of irradiated solution of 25 mL and a solution exposure area of 53 cm^2.

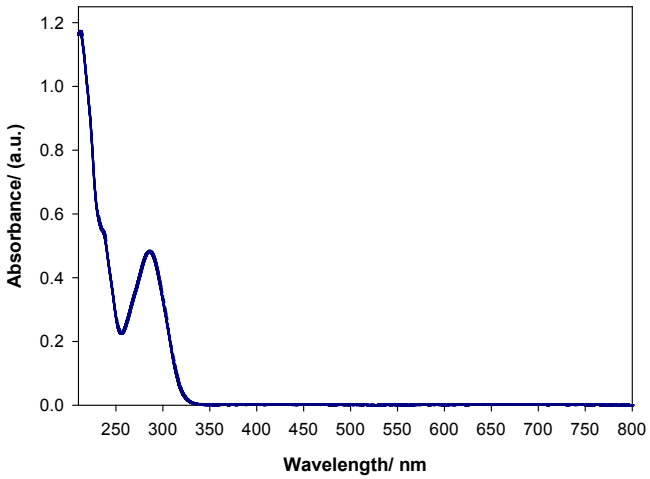

Figure 3.7. Absorbance spectrum of carbamazepine (c = 3.945 × 10^{-5} mol L^{-1}). No differences were found in the absorption spectra of carbamazepine at the four studied pH values (2.9, 4.0, 5.8 and 9.0). The spectrum was obtained using a UV-Vis Shimadzu spectrophotometer and an optical path length of 1 cm.

The quantum yield values obtained for all the experimental conditions are presented in Table 3.3 and varied between 2.0×10^{-6} and 3.5×10^{-4}, being consistent with previously published literature data (Andreozzi et al., 2003; Lam and Mabury, 2005; Chiron et al., 2006). In comparison with available data for other pharmaceuticals (Arnold and McNeill, 2007), the results here obtained highlight that carbamazepine has one of the lowest quantum yields under environmentally relevant conditions.

3.3.5 Environmental relevance of the results

The results presented in Table 3.3 (half-life times and apparent pseudo-first order rate constants) are related to specific experimental conditions: samples were irradiated with an average irradiance of 55 W m^{-2} in the range 290-400 nm (or 550 W m^{-2} in the range 290-800 nm, according to the manufacturer specifications). As the adopted lamp aims to simulate sunlight, it is reasonable to convert the obtained results to outdoor half-life times. Vione et al. (2006) and Minero et al. (2007) had shown that on a cloudless summer day (15th July, 45 ºN latitude) the total energy reaching the ground is 7.5×10^{5} J m^{-2} (290-400 nm), by measuring the solar irradiance at the ground level. These measurements were made using the same multimeter model from Co.fo.me.gra. Therefore, it is possible to convert the half-life times of carbamazepine in units equivalent to summer sunny days: the total energy reaching the ground during 24 h under the referred conditions are equivalent to 3.8 h of irradiance under the conditions adopted in this study. Thus, results obtained at pH values considered environmentally relevant to surface waters (5.8 and 9.0) were converted to equivalents to summer sunny days (SSD) and are also presented in Table 3.3. Note that this conversion takes into account the day/night cycle, as explained by Vione et al. (2006). With this approach it is possible to estimate the real behavior of carbamazepine in the environment. According to the obtained set of results and only considering direct photodegradation, this pharmaceutical can persist in the environment between 4.5 and 25 summer cloudless days, depending on the surface water pH and its level of aeration. These results are of great significance to assess the persistence of this pharmaceutical, for example, in atmosphere open aeration tanks, commonly found in urban WWTPs, where pH values usually oscillate between 6 and 9.

In general, considering only the contribution of direct photodegradation processes for the elimination of carbamazepine from aquatic environments exposed to sunlight, it could be reasonably expected that carbamazepine would take approximately 1 to 4 weeks of sunny weather to be eliminated.

3.3.6 Identification of photodegradation products by mass spectrometry

Various photodegradation products of carbamazepine were identified by mass spectrometry (compounds I-VII, Figure 3.8). The samples were analyzed by ESI(+)MS without a prior separation process, using not sparged samples collected after 24 h of irradiation at pH 5.8 (sample A) and N_2-sparged samples collected after 30 min of irradiation at pH 2.9 (sample B). The mass spectra of the selected samples were compared with a carbamazepine solution not exposed to simulated solar radiation and the ions $[M+H]^+$ and/or $[M+Na]^+$ of possible photodegradation products were identified in both samples. Subsequently, the structure of the photoproducts was tentatively assigned to each ion based on the fragmentation pathway observed in the ESI-MS^2 spectra (results reported in Table 3.4). Some examples of ESI-MS and ESI-MS^2 spectra of carbamazepine before and after irradiation are shown in Figure 3.9.

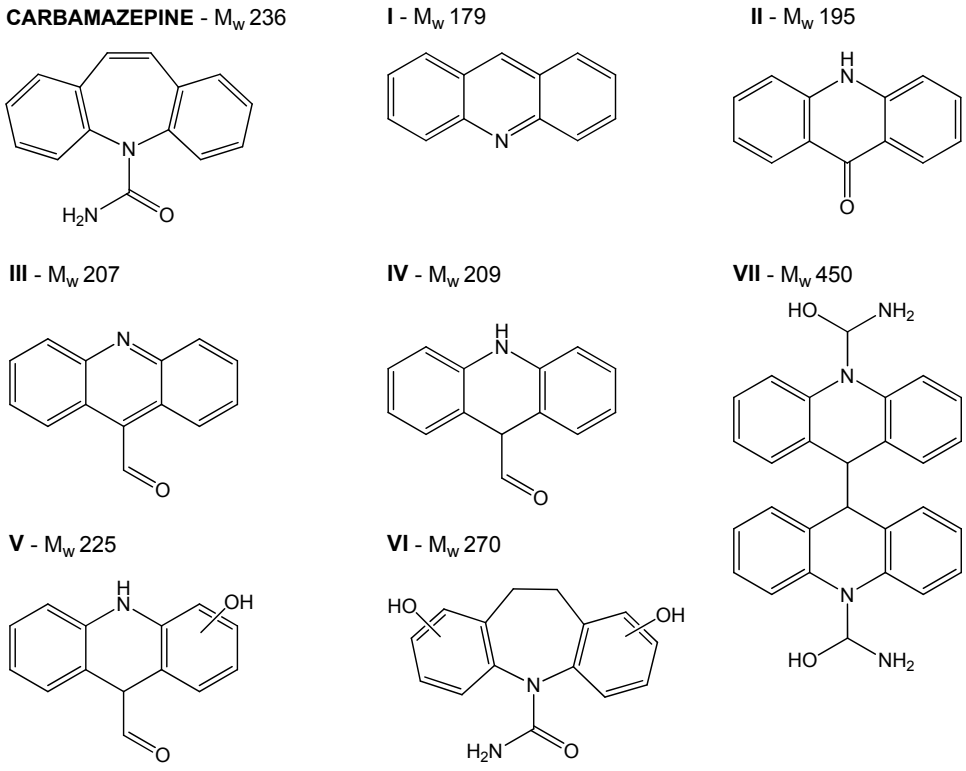

Figure 3.8. Proposed structures for the photodegradation products of carbamazepine identified by ESI(+)MS and MS^2.

Table 3.4. Mass spectrometry data for the identification of carbamazepine photodegradation products: molecular weight (M_w), ions detected in ESI(+)MS and fragment ions detected in ESI(+)MS^2. For each case, relative abundance of the relevant fragment ions and respective losses are also presented. The identification of the photoproducts using roman numbers I to VII is in accordance with Figure 3.8.

Compound	M_w	ESI(+)MS *m/z*	ESI(+)MS^2 *m/z* (relative abundance %, loss)	Samples
CBZ	236	237 $[M+H]^+$	220(2, -NH_3) 194 (100, -NHCO) 179 (2, -NHCO and -NH) 165 (1, -NHCO, -NH and -CH_2)	-
I	179	180 $[M+H]^+$	152 (80, -H_2CN) 128 (10)	A* and B**
II	195	196 $[M+H]^+$	195 (25, -H) 180(3, -NH_2) 168 (15, -CO) 167 (100, -HCO)	B
III	207	208 $[M+H]^+$	180 (100, -CO) 179 (25, - HCO) 178 (20, -H_2CO) 152 (10, -H_2CN and -CO)	B >> A
IV	209	210 $[M+H]^+$	182 (85, -CO) 180 (100, -H_2CO)	B >> A
V	225	226 $[M+H]^+$	208(90, -H_2O) 180(93, -H_2O and CO) 182(100, -$CHOHCH_2$)	B
VI	270	293 $[M+Na]^+$	276 (3, -NH_3) 275 (8, -H_2O) 250 (28, -NHCO) 248 (25, - $COHNH_2$) 232 (100, -NHCO and -H_2O)	A and B
VII	450	473 $[M+Na]^+$	456 (10, -NH_3) 439 (28,-NH_3 and -NH_3) 428 (48, -$COHNH_2$) 413 (60, - $COHNH_2$ and -NH) 411 (100, - $COHNH_2$ and -NH_3) 383 (15,- $COHNH_2$ and -$COHNH_2$)	B >> A

*Sample A: CBZ solution irradiated during 24 h, 55 W m^{-2} (290-400 nm), in ultra-pure water;

**Sample B: CBZ solution irradiated during 30 min, 55 W m^{-2} (290-400 nm), in ultra-pure water sparged with N_2, pH 2.9 (pH adjusted with formic acid).

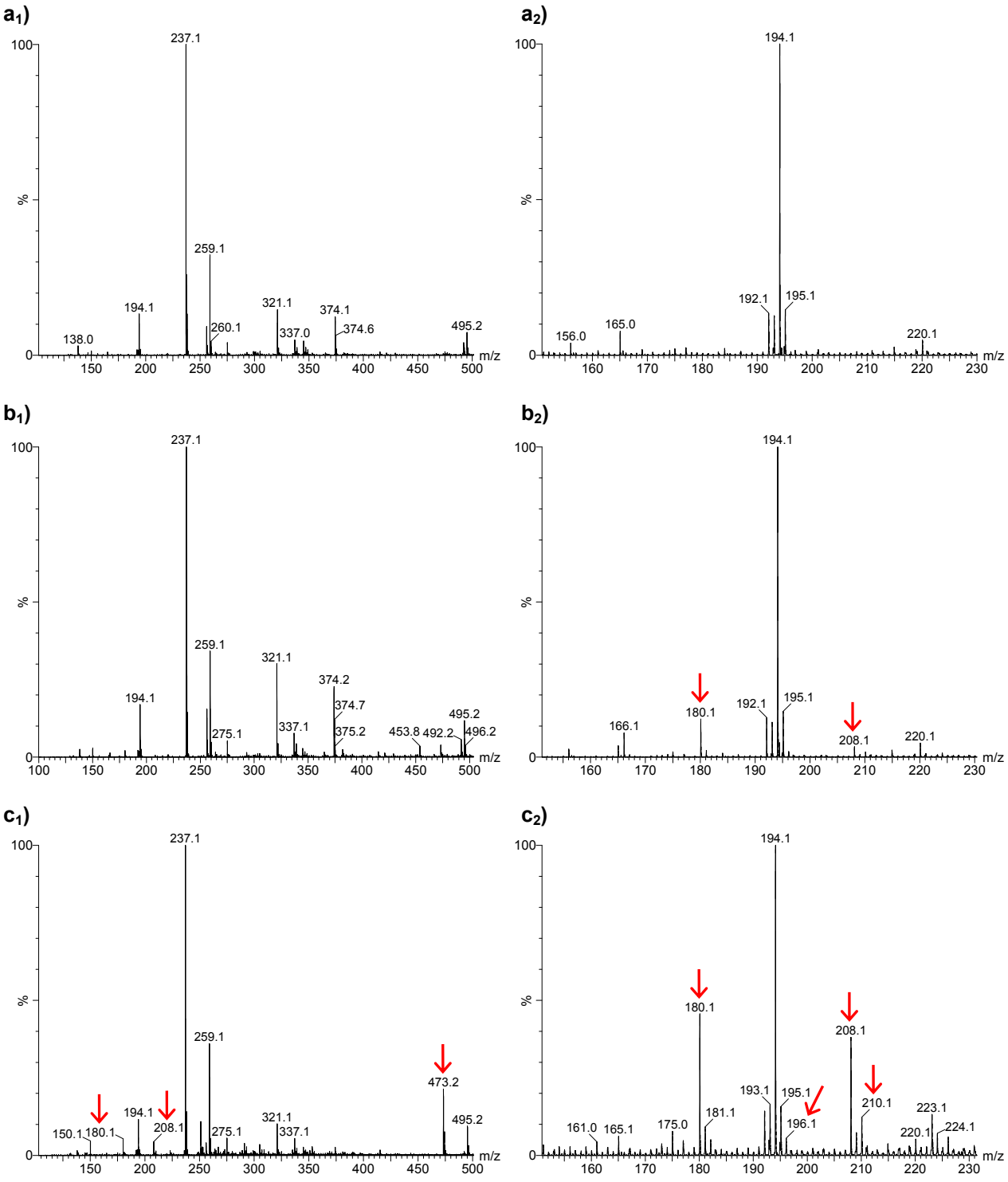

Figure 3.9. ESI-MS spectra of *a_1)* carbamazepine standard solution, *b_1)* carbamazepine solution irradiated during 24 h, in ultra-pure water and *c_1)* carbamazepine solution irradiated during 30 min, in ultra-pure water sparged with N_2, pH 2.9. *a_2)*, *b_2)* and *c_2)* are detailed views of *a_1)*, *b_1)* and *c_1)*, respectively. Red arrows draw attention to *m/z* values which correspond to photodegradation products.

As far as photodegradation product I (M_w 179, $[M+H]^+$ = 180) is concerned, ESI-MS and ESI-MS^2 spectra patterns are consistent with acridine, one of the most common carbamazepine photoproducts, known to have mutagenic and carcinogenic activities. The $[M+H]^+$ ion of photoproduct II (M_w 195) at *m/z* 196 could correspond to acridone or 9-hydroxy-acridine. Nevertheless, this product was identified as acridone seeing that its ESI-MS^2 spectrum is characterized by a base peak at *m/z* 167 that could be assigned to the loss of a CO group (-28 Da) and it is not possible to observe the loss of a water molecule (-H_2O, -18 Da) characteristic of hydroxylated products. The ESI-MS^2 spectrum of the photodegradation product III (M_w 207, $[M+H]^+$ = 208) allows identifying it as acridine-9-carbaldehyde. The increase of the molecular weight by 28 units in comparison to acridine and the base peak at *m/z* 180 is fully consistent with an acridine moiety containing a carbonyl group. Other alternatives for photodegradation products with the same molecular weight were not supported by the ESI-MS^2 spectrum. Photoproduct IV (M_w 209, $[M+H]^+$ = 210) is structurally similar to photoproduct III. The increase of the molecular weight by two units corresponds to two hydrogen atoms. Photoproduct V corresponds to a hydroxylation of compound IV; the ESI-MS^2 spectrum pattern is similar to the one found for compound IV with an extra ion at *m/z* 208 that arises from the loss of a water molecule, which reinforces the presence of the OH group in the proposed structure. Compound VI was attributed to one of the possible dihydroxicarbamazepine isomers. The ESI-MS^2 spectrum of VII (M_w 450, $[M+Na]^+$ = 473) is characterized by the loss of one and two NH_3 groups (*m/z* 456 and 439), and also the loss of one and two $COHNH_2$ groups (*m/z* 428 and 383); the ion attributed to the base peak corresponds to a combined loss of one group NH_3 and one group $COHNH_2$ (at *m/z* 411).

The proposed identification of carbamazepine's photodegradation products was further validated by exact mass measurement and elemental composition determination of the new ions, observed in the ESI-MS spectra. For this purpose, the exact calculated monoisotopic masses, corresponding to the suggested molecular formulas, were compared with the masses observed in the ESI-MS spectra. Detailed results for each photodegradation product are gathered in Table 3.5. The errors between observed and calculated masses are below 10 mDa for the majority of the photoproducts which confirms with good accuracy the presented molecular formulas.

Overall, the identified carbamazepine´s photodegradation products I-III and V-VII are in accordance with previously published studies (Chiron et al., 2006; Kosjek et al., 2009). However, photoproduct IV has not been identified until now. It is also important to highlight that the two distinct analyzed experimental conditions differed on the presence of products II and V that were not detected in sample A but appeared in sample B. In addition, experimental conditions applied to sample B resulted on the appearance of products III, IV and VII in more significant quantities than in sample A.

Table 3.5. Calculated and observed mass, mass error and double bond equivalents (DBE) for the predicted formulas of the identified photodegradation products of carbamazepine. Predicted formulas correspond to the $[M+H]^+$ ions of compounds I – V and to the $[M+Na]^+$ ions of compounds VI and VII. The designation of the photodegradation products is in accordance with Figure 3.8.

Compound	Predicted Formula	Calculated mass/ Da	Observed mass/ Da	Error/ mDa	Error/ ppm	DBE
I	$C_{13}H_{10}N$	180.0813	180.0877	6.4	35.4	9.5
II	$C_{13}H_{10}NO$	196.0762	196.0870	10.8	54.9	9.5
III	$C_{14}H_{10}NO$	208.0762	208.0835	7.3	34.9	10.5
IV	$C_{14}H_{12}NO$	210.0919	210.0983	6.4	30.5	9.5
V	$C_{14}H_{12}NO_2$	226.0868	226.0956	8.8	38.9	9.5
VI	$C_{15}H_{14}N_2O_3Na$	293.0902	293.0865	-3.7	-12.7	9.5
VII	$C_{28}H_{26}N_4O_2Na$	473.1953	473.2119	16.6	35.0	17.5

3.4 CONCLUSIONS

This study has shown that the direct photodegradation rate of carbamazepine is pH dependent. Low pH results in increased rates, while high pH is compatible with very slow degradation processes. Moreover, especially at acidic pH, it was clearly shown that the oxygenation level of waters also has a noteworthy influence on the process: the presence of oxygen is responsible for a significant decrease on the photodegradation rate of carbamazepine. Despite the fact that the extremely high photodegradation rates were obtained at pH values that are not significant in an environmental context, this study highlighted new aspects of the direct photodegradation of this pharmaceutical that have not been explored until now, particularly the role played by oxygen on the photodegradation. However, there is still much to be understood, especially concerning the mechanisms involved in the pH dependent photodegradation and in the inhibition of photodegradation driven by the presence of oxygen.

Considering the studied environmentally relevant pH conditions (5.8 and 9.0), the photodegradation rate of carbamazepine is relatively slow. The elimination of this pharmaceutical by direct photodegradation processes could take from 4.5 to 25 equivalents to sunny summer days. These results clearly consolidate previous knowledge that this pharmaceutical is being potentially accumulated in the environment and are fully consistent with the high number of occurrences of

this compound in several environmental matrices. Furthermore, the results here presented also contribute to strengthen the adequacy of carbamazepine as a marker of anthropogenic pollution due to its high persistence to direct photodegradation.

In this study, it was also possible to identify seven photodegradation products, including acridine (known due to its mutagenic and carcinogenic activities) and one newly identified compound.

Overall, the described aspects of carbamazepine degradation constitute a helpful tool to develop further investigation in what concerns the environmental persistence and relevant elimination methodologies of this widely spread contaminant.

3.5 REFERENCES

Anbar, M., Hart, E.J., **1964**. The reactivity of aromatic compounds toward hydrated electrons. *J. Am. Chem. Soc.* 86, 5633.

Andreozzi, R., Raffaele, M., Nicklas, P., **2003**. Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environment. *Chemosphere* 50, 1319-1330.

Arnold, W.A., McNeill, K., **2007**. Transformation of pharmaceuticals in the environment: photolysis and other abiotic processes. in: Petrovic, M., Barceló, D. (Eds.). *Analysis, Fate and Removal of Pharmaceuticals in the Water Cycle*. Elsevier, Amsterdam, The Netherlands.

Boreen, A.L., Arnold, W.A., McNeill, K., **2003**. Photodegradation of pharmaceuticals in the aquatic environment: A review. *Aquat. Sci.* 65, 320-341.

Boule, P., Bahnemann, D.W., Robertson, P.K.J. (Eds.), **2005**. *Environmental Photochemistry - Part II.* Springer-Verlag, Berlin.

Braslavsky, S.E., **2007**. Glossary of terms used in photochemistry. *Pure Appl. Chem.* 79, 293-465.

Chiron, S., Minero, C., Vione, D., **2006**. Photodegradation processes of the antiepileptic drug carbamazepine, relevant to estuarine waters. *Environ. Sci. Technol.* 40, 5977-5983.

Clara, M., Strenn, B., Kreuzinger, N., **2004**. Carbamazepine as a possible anthropogenic marker in the aquatic environment: investigations on the behaviour of carbamazepine in wastewater treatment and during groundwater infiltration. *Water Res.* 38, 947-954.

Erny, G.L., Rodrigues, J.E.A., Gil, A.M., Barros, A.S., Esteves, V.I., **2009**. Analysis of non-aromatic organic acids in beer by CE and direct detection mode with diode array detection. *Chromatographia* 70, 1737-1742.

Glassmeyer, S., Kolpin, D.W., Furlong, E.T., Focazio, M.T., **2008**. Environmental presence and persistence of pharmaceuticals: an overview. in: Aga, D.S. (Ed.). *Fate of Pharmaceuticals in the Environment and in Water Treatment Systems*. CRC Press, Taylor & Francis Books, Boca Raton, pp. 3-51.

Guerard, J.J., Chin, Y.-P., Mash, H., Hadad, C.M., **2009**. Photochemical fate of sulfadimethoxine in aquaculture waters. *Environ. Sci. Technol*. 43, 8587-8592.

J.N. Miller, Miller, J.C., **2005**. *Statistics and Chemometrics for Analytical Chemistry*, 5th ed. Prentice Hall, Dorset England.

Jones, O.A., Voulvoulis, N., Lester, J.N., **2002**. Aquatic environmental assessment of the top 25 English prescription pharmaceuticals. *Water Res.* 36, 5013-5022.

Joschek, H.-I., Grossweiner, L.I., **1966**. Optical generation of hydrated electrons from aromatic compounds. II. *J. Am. Chem. Soc.* 88, 3261.

Kellmann, A., Tfibel, F., **1980**. Laser-induced two-photon ionization of acridine in aqueous solution. *Chem. Phys. Lett.* 69, 61-65.

Kellmann, A., Tfibel, F., **1982**. Radicals produced from the laser-induced photoionization of acridine in solution. *J. Photochem.* 18, 81-88.

Kosjek, T., Andersen, H.R., Kompare, B., Ledin, A., Heath, E., **2009**. Fate of carbamazepine during water treatment. *Environ. Sci. Technol*. 43, 6256-6261.

Kuhn, H.J., Braslavsky, S.E., Schmidt, R., **2004**. Chemical actinometry. *Pure Appl. Chem.* 76, 2105-2146.

Kummerer, K. (Ed.), **2008**. *Pharmaceuticals in the Environment: Sources, Fate, Effects and Risks*. Springer-Verlag, Berlin.

Lam, M.W., Mabury, S.A., **2005**. Photodegradation of the pharmaceuticals atorvastatin, carbamazepine, levofloxacin, and sulfamethoxazole in natural waters. *Aquat. Sci.* 67, 177-188.

Marina, M.L., Ríos, A., Valcárcel, M. (Eds.), **2005**. *Analysis and Detection by Capillary Electrophoresi*s. Elsevier, Amsterdam, The Netherlands.

Minero, C., Chiron, S., Falletti, G., Maurino, V., Pelizzetti, E., Ajassa, R., Carlotti, M., Vione, D., **2007**. Photochemincal processes involving nitrite in surface water samples. *Aquat. Sci.* 69, 71-85.

NASA, **2008**. http://mirador.gsfc.nasa.gov (Accessed March 2011).

Neamtu, M., Frimmel, F.H., **2006**. Photodegradation of endocrine disrupting chemical nonylphenol by simulated solar UV-irradiation. *Sci. Total Environ.* 369, 295-306.

Nghiem, L.D., Schafer, A.I., Elimelech, M., **2005**. Pharmaceutical retention mechanisms by nanofiltration membranes. *Environ. Sci. Technol.* 39, 7698-7705.

OECD, **2008**. *Phototransformation of Chemicals in Water - Direct Photolysis*. Guideline for the Testing of Chemicals nº 316, Paris.

Petrovic, M., Barceló, D. (Eds.), **2007**. *Analysis, Fate and Removal of Pharmaceuticals in the Water Cycle*. Elsevier, Amsterdam, The Netherlands.

Pranaityté, B., Padarauskas, A., **2006**. Characterization of the SDS-induced electroosmotic flow in micellar electrokinetic chromatography with cationic polyelectrolyte-coated capillaries. *Electrophoresis* 27, 1915-1921.

Pucci, V., Raggi, M.A., **2005**. Analysis of antiepileptic drugs in biological fluids by means of electrokinetic chromatography. *Electrophoresis* 26, 767-782.

Schwarzenbach, R.P., Gschwend, P.M., Imboden, D.M., **2003**. *Environmental Organic Chemistry*. John Wiley & Sons, New Jersey.

Sevilla-Morán, B., Mateo-Miranda, M.M., Alonso-Prados, J.L., García-Baudín, J.M., Sandín-España, P., **2010**. Sunlight transformation of sethoxydim-lithium in natural waters and effect of humic acids. *Int. J. Environ. An. Ch.* 90, 487 - 496.

Skoog, D.A., Holler, F.J., Nieman, T.A., **1998**. *Principles of Instrumental Analysis*, 5th ed. Saunders College Publishing.

Smyth, W.F., McClean, S., **1998**. A critical evaluation of the application of capillary electrophoresis to the detection and determination of 1,4-benzodiazepine tranquilizers in formulations and body materials. *Electrophoresis* 19, 2870-2882.

Terabe, S., Otsuka, K., Ando, T., **1985**. Electrokinetic chromatography with micellar solution and open-tubular capillary. *Anal. Chem.* 57, 834-841.

Terabe, S., Otsuka, K., Ichikawa, K., Tsuchiya, A., Ando, T., **1984**. Electrokinetic separations with micellar solutions and open-tubular capillaries. *Anal. Chem.* 56, 111-113.

Ternes, T.A., Herrmann, N., Bonerz, M., Knacker, T., Siegrist, H., Joss, A., **2004**. A rapid method to measure the solid-water distribution coefficient (K_d) for pharmaceuticals and musk fragrances in sewage sludge. *Water Res.* 38, 4075-4084.

Thormann, W., Theurillat, R., Wind, M., Kuldvee, R., **2001**. Therapeutic drug monitoring of antiepileptics by capillary electrophoresis: Characterization of assays via analysis of quality control sera containing 14 analytes. *J. Chromatogr. A* 924, 429-437.

Vanhoenacker, G., de l'Escaille, F., De Keukeleire, D., Sandra, P., **2004**. Analysis of benzodiazepines in dynamically coated capillaries by CE-DAD, CE-MS and CE-MS^2. *J. Pharmaceut. Biomed.* 34, 595-606.

Vione, D., Falletti, G., Maurino, V., Minero, C., Pelizzetti, E., Malandrino, M., Ajassa, R., Olariu, R.-I., Arsene, C., **2006**. Sources and sinks of hydroxyl radicals upon irradiation of natural water samples. *Environ. Sci. Technol.* 40, 3775-3781.

Weinberger, R., **2000**. *Practical Capillary Electrophoresis*, 2nd ed. Academic Press, New York.

Yu, L., Fink, G., Wintgens, T., Melin, T., Ternes, T.A., 2009. Sorption behavior of potential organic wastewater indicators with soils. *Water Res.* 43, 951-960.

Zafiriou, O.C., Joussot-Dubien, J., Zepp, R.G., Zika, R.G., **1984**. Photochemistry of natural waters. *Environ. Sci. Technol.* 18, 358A-371A.

Zepp, R.G., Braun, A.M., Hoigne, J., Leenheer, J.A., **1987**. Photoproduction of hydrated electrons from natural organic solutes in aquatic environments. *Environ. Sci. Technol.* 21, 485-490.

CHAPTER 4

Photodegradation of benzodiazepines in aquatic environments

In this study, the relevance of photodegradation processes on the environmental persistence of four benzodiazepines (oxazepam, diazepam, lorazepam and alprazolam) was investigated. Benzodiazepines were irradiated under simulated solar irradiation and direct and indirect (together with three different fractions of humic substances) photodegradation kinetics were determined. Lorazepam was shown to be quickly eliminated by direct solar radiation; on the contrary, oxazepam, diazepam and alprazolam showed to be highly resistant to photodegradation with half-life times of 4, 7 and 228 sunny summer days, respectively. Humic acids were consistently responsible for a decrease in the photodegradation rates while fulvic acids and XAD4 fraction caused an enhancement of the photodegradation rate. Overall, the results highlight that photodegradation might not be an efficient pathway to prevent the aquatic environmental accumulation of oxazepam, diazepam and alprazolam. Also, nineteen direct photodegradation products were identified by mass spectrometry; this identification is crucial for a more complete understanding of the environmental impact of benzodiazepines in aquatic systems.

CONTENTS

4.1 CONTEXTUALIZATION

Benzodiazepines belong to the group of psychiatric substances which act on the central nervous system, having anxiolytic, sedative and hypnotic effects and are one of the most prescribed groups of pharmaceuticals throughout the world (International Narcotics Control Board, 2010; Health, 2011).

Literature data presented in chapter 2 clearly demonstrate that, over the last decade, there have been a significant number of studies reporting the occurrence of benzodiazepines in environmental matrices. However, and when compared to other groups of pharmaceuticals, data concerning the environmental persistence and fate of benzodiazepines is scarce. In order to correctly evaluate the real ecological impact of these pollutants, it is mandatory to widen the knowledge related to this area of concern.

The work presented in this chapter intends to approach, for the first time, to the extent of our knowledge, the persistence of benzodiazepines in aquatic environments. For this purpose, the photodegradation of four benzodiazepines (oxazepam, diazepam, lorazepam and alprazolam) was studied. Direct and indirect photodegradation (in the presence of different fractions of humic substances: humic acids, fulvic acids and XAD4 fraction) were evaluated under simulated solar irradiation. To follow the photodegradation kinetics of these compounds, the micellar electrokinetic chromatography method developed to study carbamazepine photodegradation (presented in the previous chapter) was adapted. Moreover, taking into account that the available literature studies about the photodegradation products of benzodiazepines are not environmentally relevant, focusing only on drug-development, storage and handling of these pharmaceuticals (Cabrera et al., 2005; Castaneda et al., 2009), this study also aims to be the first at identifying direct photodegradation products of the four benzodiazepines by mass spectrometry, under environmentally relevant conditions.

Some of the main concepts essential to the understanding of the work here presented can be found in the previous chapter (section 3.1). A brief introduction to the topics that will be addressed for the first time is presented below.

4.1.1 Indirect photodegradation

In the previous chapter the importance of direct photodegradation was studied. There are, however, other environmentally relevant light initiated reactions that do not result from the direct

absorption of photons by pollutants (Schwarzenbach et al., 2003). These are considered indirect photodegradation processes and the phototransformation is indirectly caused by the excitation of chromophores present in natural waters (Boule et al., 2005). In opposition to direct photodegradation, the efficiency of indirectly mediated phototransformations does not depend on the absorption spectrum of the contaminant and can play an important role on the degradation of pollutants that poorly absorb solar radiation or that resist to direct photolysis. The relevance of indirect photodegradation is then dependent on the number and type of reactive species produced by the excitation of chromophores and on their subsequent ability to react and cause a chemical change in a specific contaminant (Zafiriou et al., 1984; Boule et al., 2005). In comparison with molecular oxygen, these reactive species are usually strong oxidants; photoreduction in natural waters is also possible but it is less common to occur. These photo-oxidants are commonly short-lived species which can be quickly deactivated by psychical or chemical processes and commonly include $HO^{\cdot}$, $^{1}O_2$, alkyl-$O^{\cdot}$, alkyl-$OO^{\cdot}$, aryl-$O^{\cdot}$, $O_2^{\cdot -}$, $NO_3^{\cdot}$, with reactive oxygen species playing a major role. Amongst the natural constituents that may yield these reactive species, the most well known are dissolved organic matter, nitrates, nitrites and Fe(II)/ Fe(III) complexes (Schwarzenbach et al., 2003).

Although direct photodegradation studies are of extreme importance, they do not give enough information concerning the environmental persistence of a contaminant. The photochemical fate of a pollutant might differ significantly when in presence of naturally occurring constituents which can have light absorbing, quenching or sensitizing effects (Zepp et al., 1985; Zeng et al., 2002). The study of indirect photodegradation under environmentally relevant conditions can be performed by using representative natural water samples or synthetic field water (produced in laboratory). The use of natural water samples has the advantage of providing an authentic reproduction of field conditions. On the other hand, the use of synthetic field waters allows testing the individual role and influence of each component in the photodegradation (Arnold and McNeill, 2007).

4.1.2 The role of dissolved organic matter on indirect photodegradation

Dissolved organic matter (DOM) is the most abundant fraction of natural organic matter in aquatic environments and result from the metabolism or decomposition of living organisms (Boule et al., 2005). The main constituents of DOM are humic substances which can be divided in several fractions, classified according to their solubility in water (such as humic acids, fulvic acids,

hydrophilic fractions - XAD4 fraction - and humins). DOM is involved in a huge number of environmental processes, including photochemical reactions. For this reason, the study of DOM characteristics and effects leads to a better understanding of important environmental phenomena (Leenheer and Croué, 2003).

DOM can be defined as a complex heterogeneous mixture of aromatic and aliphatic hydrocarbons that possess a large variety of functional groups (mainly, carboxyl, hydroxyl, ester, ether and ketone groups). These relatively small primary molecular structures (100 – 2000 Da) are held together by hydrogen bonding, non-polar and cation interactions resulting in aggregates with macromolecular characteristics, with up to 100 000 Da of molecular weight (Leenheer and Croué, 2003; Boule et al., 2005). DOM composition, concentration and characteristics are highly variable according to the provenience of the organic matter (allochthonous or autochthonous) and to the physical and chemical properties of water (Leenheer and Croué, 2003). Differences on the DOM source and composition usually imply differences on its photoreactivity (Boreen et al., 2008; Guerard et al., 2009). DOM, and particularly, humic substances are responsible for the yellow or brown color of natural waters. This fact underlines the light absorbing ability of DOM and its consequent photochemical activity (Schwarzenbach et al., 2003). Some of the main photochemical processes mediated by DOM are summarized in Figure 4.1.

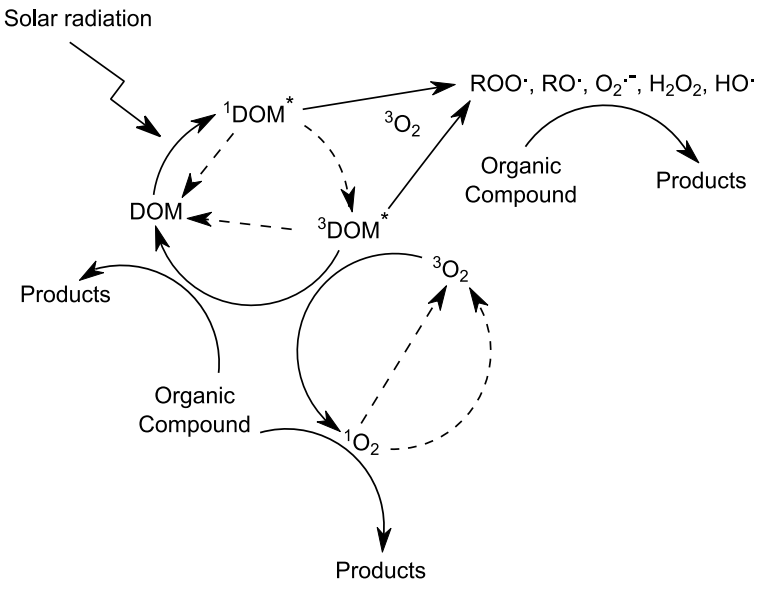

Figure 4.1. Main pathways for indirect photodegradation of an organic compound caused by excitation of naturally occurring dissolved organic matter (DOM). Dashed arrows represent radiationless transitions. Adapted from Zafiriou et al. (1984) and Schwarzenbach et al. (2003).

After absorbing a photon, DOM chromophores are promoted to their excited singlet state which, in the presence of molecular oxygen, can lead to the formation of singlet oxygen and other reactive oxygen species (Boule et al., 2005). The same phenomenon is possible due to the formation of excited triplet states of DOM chromophores ($^3DOM^*$). The produced reactive oxygen species include DOM derived oxyl and peroxyl radicals ($ROO^{\cdot}$, $RO^{\cdot}$), superoxide radical anions ($O_2^{\cdot -}$), hydroxyl radicals ($HO^{\cdot}$) and hydrogen peroxide (H_2O_2). These reactive species are then responsible for promoting indirect photodegradation by interacting with the contaminants (Zafiriou et al., 1984; Zepp et al., 1985; Guerard et al., 2009; Dalrymple et al., 2010). Direct reactions with excited triplet states of DOM are also possible through energy transfer (which may cause, for example, the cis-trans isomerization of double bonds) or electron/hydrogen transfer reactions which lead to the oxidation of the contaminants (Schwarzenbach et al., 2003). However, the effect of DOM on the photodegradation is not trivial: DOM chromophores can also act as inhibitors by scavenging the reactive intermediates mentioned and by screening the radiation (Zepp et al., 1985; Boule et al., 2005; Xia et al., 2009; Sturini et al., 2010). The evaluation of DOM effects in the aquatic environmental persistence of organic pollutants is a rather complex issue that is strictly dependent on DOM properties, concentration and chemical structure of the contaminants.

4.2 MATERIALS AND METHODS

4.2.1 Chemicals

All chemicals used were of analytical grade: alprazolam, lorazepam, diazepam e oxazepam (Sigma-Aldrich), sodium dodecylsulphate (SDS, 99%, for electrophoresis, Sigma-Aldrich), hexadimethrine bromide (polybrene, Sigma-Aldrich), sodium chloride, ethylvanillin (99%, Aldrich), sodium tetraborate (borax, Riedel-de Haën), sodium hydroxide (Fluka), and acetonitrile (HPLC gradient grade, VWR, Prolabo). All solutions were prepared using ultra-pure water, obtained from a Milli-Q Millipore system (Milli-Q plus 185).

4.2.2 Irradiation experiments

4.2.2.1 Irradiation apparatus

Samples were irradiated under simulated solar radiation using Solarbox 1500 (Co.fo.me.gra, Italy). The irradiation device was equipped with a 1500 W arc xenon lamp and

outdoor UV filters that restrict the transmission of light with wavelengths below 290 nm (a schematic representation of the irradiation chamber is shown in Figure 3.2). The irradiance of the lamp was kept constant during all the experiments at 55 W m^{-2} (290-400 nm). To monitor the irradiance level and temperature, a multimeter (Co.fo.me.gra, Italy), equipped with a UV 290-400 nm large band sensor and a black standard temperature sensor, was used. The device was refrigerated by an air cooled system and the uniformity of the irradiation inside the chamber was guaranteed by a parabolic reflection system.

4.2.2.2 Sample preparation and sampling

Irradiated samples consisted on benzodiazepines' aqueous solutions with a concentration of 10 mg L^{-1}. Due to the low solubility of the studied compounds in water, acetonitrile was used as an auxiliary solubilising agent. The concentration of acetonitrile did not exceed 1% (*v/v*) of the irradiated solution, in order to guarantee that acetonitrile does not influence the photodegradation rates, as recommended by the OCDE guideline TG316 (OECD, 2008). The pH of the irradiated solutions was not adjusted and samples were not buffered in order to avoid influences of the buffering agent in the photodegradation process. Solutions were irradiated in triplicate using 25 mL quartz tubes with an internal diameter of 1.5 cm. For each set of experiments, dark controls were also irradiated, in triplicate; for this purpose, quartz tubes were covered with several layers of aluminum foils. A homemade metallic holder was used to maintain the quartz tubes suspended inside the irradiation chamber, allowing for homogeneous irradiation.

The extent of the irradiation experiments depended on the phototransformation rates of each pharmaceutical, varying between 12 and 304 h. 2 mL aliquots of the irradiated samples (and dark controls) were collected at specific time intervals, stored at 4 ºC and subsequently analyzed by MEKC, within 2 days, as described below.

4.2.2.3 Influence of dissolved organic matter on photodegradation

To study the influence of different fractions of dissolved organic matter on the photodegradation rates of benzodiazepines, solutions containing 1 mg L^{-1} of humic acids, fulvic acids or XAD4 fraction were irradiated in the conditions described above. For this purpose, fulvic acids and XAD4 solutions (20 mg L^{-1}) were prepared in ultra-pure water. In the case of humic acids, due to the low solubility in water, an aqueous solution (50 mg L^{-1}) 5% (*v/v*) NH_4OH 1 M was prepared and set to pH 6.0 with formic acid 1 M. The stock solutions were further diluted (20 and

50 fold) in order to attain a final concentration of 1 mg L^{-1}, as referred above. Similarly to direct photodegradation studies, irradiated solutions were not buffered and the pH was not adjusted.

The humic substances were extracted and isolated from a riverine water sample, collected in a freshwater stream that flows into the Aveiro lagoon, Portugal (40º39'N, 8º44'W). Humic substances of this riverine aquatic system are mainly derived from the decomposition of herbaceous plants. The extraction and characterization of the humic substances had been previously performed by the research group. Briefly, the different fractions of humic substances were extracted and isolated by using a system of XAD resins (XAD8 and XAD4) connected in series. This procedure is described in detail in Santos et al. (1994) and Esteves et al. (1995). The purified fractions (humic acids, fulvic acids or XAD4) were subsequently characterized by elemental analysis and solid-state ^{13}C-CPMAS NMR, using the procedure described in Esteves et al. (2009).

4.2.3 Capillary electrophoresis

The kinetics of photodegradation was followed by MEKC. The analyses were carried out using a commercial instrument Beckman P/ACE MDQ (Fullerton, CA, USA), equipped with a photodiode array UV-Vis detector. The methodology was adapted from the work presented in chapter 3, where a MEKC method was developed to follow the kinetics and emergence of photodegradation products of the anti-epileptic carbamazepine. This method is based on the use of a dynamically coated capillary column (Figure 3.3) with the aim of improving reproducibility and separation efficiency.

4.2.3.1 Capillary column conditioning, coating and separation conditions

A fused-silica capillary with a total length of 50 cm (40 cm to detector) and 75 µm of internal diameter was used. Capillaries were first conditioned with 1 M NaOH for 30 min, followed by ultra-pure water for 15 min. Capillary coating was performed by flushing a solution of polybrene 0.5% (*w/v*) in 0.5 M NaCl for 20 min, followed by ultra-pure water for 2 min and running buffer for 20 min. The capillary was washed with ultra-pure water, for 5 min, at the end of each working day and with running buffer, for 20 min, at the beginning of the day. All the conditioning and coating steps were performed at 20 psi.

Samples were injected for 4 s at 0.5 psi. Separation was carried out in direct polarity with a positive potential supply of 20 kV for 13 min. The capillary temperature was controlled and

maintained at 25 ºC. Detection of benzodiazepines and its photodegradation products was monitored at 210 nm. Before every run, the capillary was washed with running buffer for 2 min. Running buffer vials were changed every 6 runs.

4.2.3.2 Running buffer, standard solutions and sample preparation for MEKC analysis

The running buffer consisted on 25 mM sodium tetraborate and 50 mM SDS, pH 9.2, freshly prepared every 3 days and stored at 4 ºC.

For the MEKC calibration curves, seven standard solutions of each benzodiazepine, with concentrations ranging between 0.5 and 10 mg L^{-1}, were prepared by diluting an aqueous stock solution with concentration of 200 mg L^{-1} (20% acetonitrile). Standard solutions also contained 3.34 mg L^{-1} ethylvanillin, used as internal standard (IS), and 12.5 mM sodium tetraborate, used to improve the repeatability of the ratio between benzodiazepines and IS peak areas. Standard solutions were analyzed in quadruplicate.

Similarly to the standard solutions, irradiated samples were prepared by adding sodium tetraborate and IS with a final concentration of 12.5 mM and 3.34 mg L^{-1}, respectively.

All solutions were filtered through a 0.22 μm membrane filters (Millex-GV, Millipore).

4.2.4 Identification of photodegradation products by mass spectrometry

The direct photodegradation products of benzodiazepines were identified by electrospray mass spectrometry (ESI-MS). Irradiated samples of each pharmaceutical were collected after 6, 24, 24 and 304 hours of irradiation for lorazepam, oxazepam, diazepam and alprazolam, respectively. The irradiation time was selected according to the half-life time of each compound and considering the time at which a larger number of photodegradation products could be clearly identified in the electrochromatogram. Positive-ion ESI-MS and ESI-MS^2 were performed on a Micromass Q-TOF2 hybrid tandem mass spectrometer (Manchester, UK). For ESI analysis, samples were prepared by diluting the irradiated solutions in methanol (0.1% formic acid *v/v*). Samples were introduced into the ESI source using a syringe pump at a flow rate of 10 μL min^{-1}. The time-of-flight (TOF) mass resolution was set to approximately 9000, the cone voltage was 35 V and the capillary voltage was

3 kV. The source and the desolvation temperatures were 80 and 150 ºC, respectively. The MS^2 spectra were acquired using argon as the collision gas and collision energy was set between 10 and 32 eV. To calibrate the MS spectra, the lock mass was the calculated monoisotopic mass/charge of the ion $[M+H]^+$ of each pharmaceutical. The data was processed using MassLynx software, version 4.0.

4.3 RESULTS AND DISCUSSION

4.3.1 Performance of the MEKC methodology

The adopted MEKC method proved to be efficient in the separation of each benzodiazepine from its photodegradation products. As concluded in the previous chapter, the use of a dynamically coated capillary column highly improved the repeatability of both migration times and peak areas since the coating avoids fluctuations due to the modification of the chemical structure of the inner surface of the capillary between successive runs. Nevertheless, there is a fundamental difference from the method used in chapter 3 that must be emphasized. In the previous work, it was concluded that the addition of running buffer to samples also improved the repeatability of the analyte peak areas. However, in this case, instead of adding the running buffer (SDS and sodium tetraborate), only sodium tetraborate was added. The removal of SDS from the samples and standard solutions resulted in a significant tighten of the peaks (as shown in Figure 4.2), thus increasing peak resolution. Separation of benzodiazepines from its photodegradation products was only achieved after introducing this modification in the sample preparation procedure, prior to MEKC analysis.

Under the optimized conditions, the migration times (from 10 repeated injections) of the studied benzodiazepines are presented in Table 4.1. Relative standard deviations did not exceed 0.55%, showing a good repeatability between successive runs. Moreover, relative standard deviations of the ratio between the analyte and the IS peak areas were always below 3%, for all benzodiazepines. A linear calibration curve was obtained for each benzodiazepine using seven standard solutions with concentrations ranging from 0.5 to 10 mg L^{-1}, analyzed in quadruplicate. The results were fitted to a least-squares linear regression by plotting the ratio between the peak area of the analyte and the peak area of the IS as a function of the analyte concentration. The statistical parameters of the obtained calibration curves are presented in Table 4.1. The obtained correlation coefficients range from 0.9997 to 0.9999, thus corroborating the excellent linear response of the method in the studied range of concentrations. The limits of detection (LOD) and limits of quantification (LOQ) were also determined, defined as $3s_{x/y}/b$ and $10s_{x/y}/b$, respectively, where b is the slope and $s_{x/y}$ is the residual standard deviation of the linear regression (J.N. Miller

and Miller, 2005). LOQs between 0.598 mg L^{-1} for alprazolam and 0.318 mg L^{-1} for diazepam allow following the kinetics of photodegradation up to, approximately, 95% of degradation of the benzodiazepines, confirming the adequacy of the methodology for this purpose.

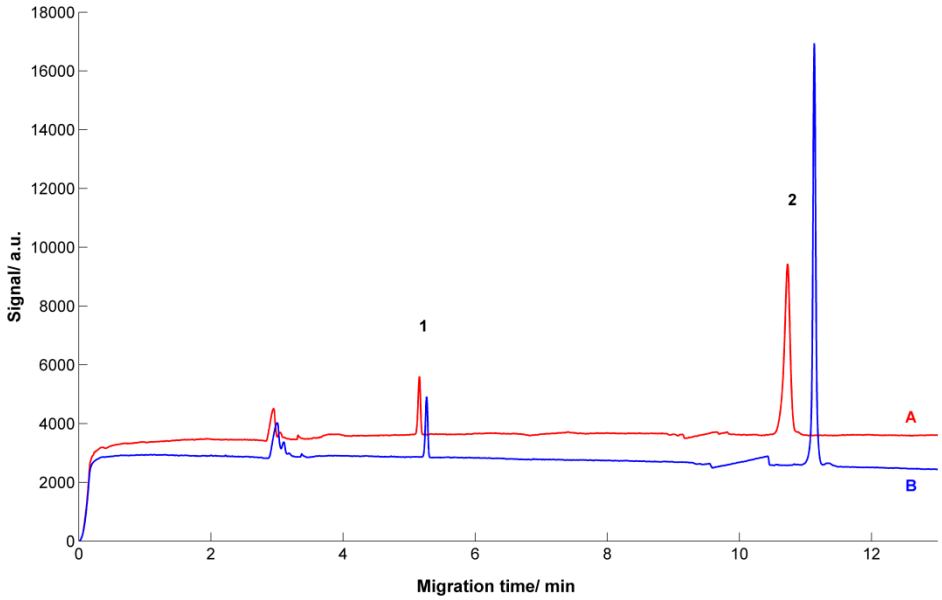

Figure 4.2. Electrochromatograms of diazepam stock solutions (10 mg L^{-1}), obtained using a coated capillary, with the addition of running buffer (SDS and sodium tetraborate) – A, and sodium tetraborate – B. Peak identification: 1 - ethylvanilin (internal standard) and 2 – diazepam; peak areas are similar in both electrochromatograms. Experimental conditions: capillary 0.5 m length (0.4 to detector), 75 µm internal diameter, applied voltage 20 kV, capillary temperature 25 ºC, running buffer 50 mM SDS and 25 mM borax, detection at 210 nm.

Table 4.1. Linear regression equation, correlation coefficient (*r*), limit of detection (LOD) and limit of quantification (LOQ) of the MEKC methodology for oxazepam, diazepam, lorazepam and alprazolam. Migration time (± standard deviation) of each pharmaceutical is also presented.

	Linear regression equation	*r*	LOD /(mg L^{-1})	LOQ /(mg L^{-1})	m_t /(min), n=10
Oxazepam	y = (0.861 ± 0.002) x - 0.005 ± 0.007	0.9999	0.103	0.343	10.94 ± 0.06
Diazepam	y = (0.917 ± 0.003) x - 0.003 ± 0.007	0.9999	0.0953	0.318	11.12 ± 0.02
Lorazepam	y = (0.886 ± 0.004) x + 0.00 ± 0.01	0.9998	0.154	0.512	10.74 ± 0.02
Alprazolam	y = (0.830 ± 0.004) x - 0.01 ± 0.01	0.9997	0.179	0.598	11.34 ± 0.03

4.3.2 Photodegradation of benzodiazepines in the presence and in the absence of dissolved organic matter

Direct and indirect photodegradation of oxazepam, diazepam, lorazepam and alprazolam were individually evaluated in aqueous solutions of 10 mg L^{-1}, with triplicate experiments, under simulated solar irradiation. Indirect photodegradation was studied in the presence of humic acids, fulvic acids and XAD4 fraction of humic substances from a freshwater sample, at a final concentration of 1 mg L^{-1}. Dark controls, obtained under the exact same conditions, were performed for all the irradiation experiments. Degradation in the dark, due to thermal or hydrolytic processes, was not observed for oxazepam, lorazepam and alprazolam; on the other hand, diazepam suffered a decrease in concentration up to 10% during 24 h. The observed degradation of diazepam in the dark may be attributed to the temperature rise inside the irradiation chamber (35 – 40 ºC) as the same phenomenon was not observed in refrigerated or room temperature solutions. Diazepam photodegradation data were corrected in order to account for non-photolytic processes occurring in samples.

The extent of the irradiation experiments depended on the photodegradation rates of each benzodiazepine, which varied from 12 to 304 h. The determination of kinetic parameters was performed by fitting a pseudo-first order kinetic model to each set of results. Accordingly, the natural logarithm of the ratio between the benzodiazepines' concentration, at a given irradiation time, and its initial concentration was plotted as a function of the irradiation time and a linear regression was obtained (Figure 4.3). Correlation coefficients (r), apparent pseudo-first order rate constants (k) and half-lives ($t_{1/2}$) are presented in Table 4.2. For both direct and indirect photodegradation, r ranges from 0.961 to 0.999, confirming the adequacy of the pseudo-first order model to describe the benzodiazepines' photodegradation kinetics.

Direct photodegradation half-lives of the studied benzodiazepines greatly differ. Lorazepam was the least photo resistant, with a half-life time of 2.6 ± 0.1 h; oxazepam and diazepam had intermediate half-life times of 15.1 ± 0.2 and 28 ± 2 h, respectively. Note that these compounds are structurally very similar (especially lorazepam and oxazepam, see Figure 4.7 ahead) and, yet, exhibit a distinct resistance to photodegradation. Alprazolam showed to be highly resistant to direct photodegradation with a half-life time of 865 ± 41 h. This value was determined by an extrapolation based on 200 h of irradiation, due to the extremely high number of irradiation hours needed (more than 1000 h). Notwithstanding, the conducted experiment is clearly conclusive about the behavior of alprazolam when exposed to sunlight. In order to determine the half-life

times of lorazepam, diazepam and oxazepam, these were irradiated until at least 75% of degradation was achieved.

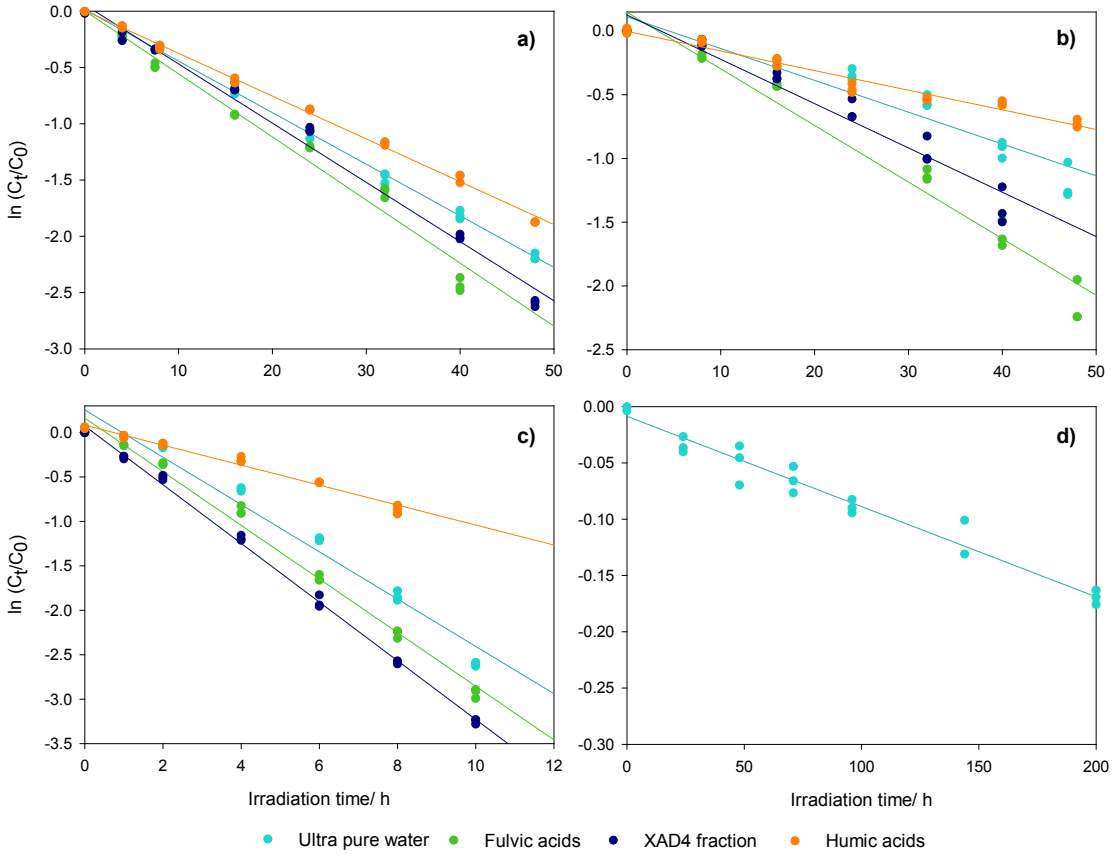

Figure 4.3. Pseudo-first order photodegradation kinetics of *a)* oxazepam, *b)* diazepam, *c)* lorazepam and *d)* alprazolam in ultra-pure water and in the presence of several fractions of humic substances. Each dot corresponds to one replicate (total of three replicates for each condition at a given time).

In what concerns indirect photodegradation, the half-life times obtained in the presence of different fractions of humic substances are of the same order of magnitude of those in pure water. For alprazolam, half-life times were not determined seeing that no photodegradation was observed after 48 h of irradiation. Considering the conclusions taken concerning alprazolam´s direct photodegradation, irradiations under indirect degradation conditions were not extended for a longer period. However, for oxazepam, diazepam and lorazepam, the addition of humic substances (1 mg L^{-1}) caused a clear and consistent influence on the photodegradation rates: humic acids (the most hydrophobic fraction) decreased the degradation rates while fulvic acids and XAD4 fraction (the most hydrophilic fractions) enhanced the photo-induced transformations. These results suggest the existence of a correlation between the hydrophobicity of the humic substances and the effect on the photodegradation rates of benzodiazepines. As stated before, the effects of humic substances on

photodegradation processes are of extreme complexity as they can exert, simultaneously, enhancing and reducing effects (production and/or scavenging of reactive intermediates and screening of reactive light wavelengths). In addition, the function of humic substances on photochemical processes is highly dependent on its concentration and chemical composition; however, a direct correlation between chemical composition and photochemical function is very often difficult to establish (Boule et al., 2005; Garbin et al., 2007; Guerard et al., 2009).

Table 4.2. Kinetic parameters for the photodegradation of oxazepam, diazepam, lorazepam and alprazolam under different experimental conditions: correlation coefficient (*r*), half-life time ($t_{1/2}$) and apparent photodegradation rate (*k*) for a pseudo-first order kinetics. *n* represents the number of experimental points used in the fitting and σ represents the standard deviation. Half-life time in units equivalent to summer sunny days (SSD) and average apparent quantum yields (φ_{ave}) are also shown (discussed in section 4.3.3).

	DOM	*r*	*n*	$k \pm \sigma/h^{-1}$	$t_{1/2} \pm \sigma$/h	$t_{1/2} \pm \sigma$/SSD	Φ_{ave}
Oxazepam	-	0.999	24	0.0458 ± 0.0005	15.1 ± 0.2	3.98 ± 0.04	4.45×10^{-6}
	Fulvic acids	0.989	21	0.056 ± 0.002	12.4 ± 0.4	3.2 ± 0.1	-
	XAD4	0.995	21	0.053 ± 0.001	13.2 ± 0.3	3.47 ± 0.08	-
	Humic acids	0.998	24	0.0380 ± 0.0004	18.2 ± 0.2	4.80 ± 0.06	-
Diazepam	-	0.961	21	0.025 ± 0.002	28 ± 2	7.3 ± 0.5	4.3×10^{-6}
	Fulvic acids	0.984	18	0.044 ± 0.002	15.6 ± 0.7	4.1 ± 0.2	-
	XAD4	0.974	18	0.035 ± 0.002	20 ± 1	5.2 ± 0.3	-
	Humic acids	0.982	21	0.0154 ± 0.0007	45 ± 2	11.8 ± 0.5	-
Lorazepam	-	0.983	18	0.27 ± 0.01	2.6 ± 0.1	0.68 ± 0.03	7.8×10^{-5}
	Fulvic acids	0.996	21	0.302 ± 0.007	2.29 ± 0.05	0.60 ± 0.01	-
	XAD4	0.999	21	0.330 ± 0.004	2.10 ± 0.02	0.552 ± 0.006	-
	Humic acids	0.991	18	0.113 ± 0.004	6.2 ± 0.2	1.62 ± 0.06	-
Alprazolam	-	0.980	20	0.00080 ± 0.00004	865 ± 41	228 ± 11	3.4×10^{-6}
	Fulvic acids	n.d.*	n.d.	n.d.	n.d.	n.d.	-
	XAD4	n.d.	n.d.	n.d.	n.d.	n.d.	-
	Humic acids	n.d.	n.d.	n.d.	n.d.	n.d.	-

* n.d.: not determined

To complement this discussion, the structural characterization of the used humic substances (Elemental Analysis and ^{13}C-CPMAS NMR data) is displayed in Tables 4.3 and 4.4 (Esteves, 1995). The presented data revealed that differences in the ^{13}C-CPMAS NMR spectra of humic substances might suggest a higher prevalence of aromatic moieties in humic acids than in fulvic acids or XAD4 (spectra range between 108-145 and 145-160 ppm). Moreover, fulvic acids and

XAD4 fractions seem to have a higher number of carboxylic and ester groups (chemical shift range 160-190 ppm) and also a higher carbon/nitrogen ratio. The influence of these compositional differences on the distinct effect of humic acids and fulvic acids/XAD4 on the photodegradation of benzodiazepines cannot be clearly defined without further research concerning the photoreaction's mechanisms. Also, it must be noted that apart from several evidences of an apparent correlation between the ^{13}C-CPMAS NMR signal strength and the abundance of the respective chemical groups, these data should always be cautiously interpreted, especially when looking for small differences (Newman, 2007) since CPMAS experiments are known to be not quantitative.

Table 4.3. Elemental analysis (in percentage) of humic substances selected to study the effect of DOM on photodegradation rates. Results are corrected for humidity at 60 ºC and ashes at 750 ºC (Esteves, 1995).

	C	H	N	S	O	Ratio C/N
Humic acids	51.4	4.3	4.2	2.1	32.1	12.2
Fulvic acids	54.0	4.8	1.9	1.5	35.2	28.4
XAD-4	49.2	4.4	2.9	1.5	40.0	17.0

Table 4.4. Solid-state ^{13}C-CPMAS NMR data of humic acids, fulvic acids and XAD4 fraction (Esteves, 1995). The data represents the area (in percentage) of the fraction of the ^{13}C-CPMAS NMR spectra due to the carbons in the specified chemical shift range (in ppm). Typical functional groups whose carbons resonate in the specified range are given in parenthesis.

Chemical shift range/ ppm	**^{13}C-CPMAS NMR spectra fraction (%)**		
	Humic acids	**Fulvic acids**	**XAD-4**
0-60 *(alkyl and methoxyl carbons)*	51.3	61.1	55.9
60-90 *(O-alkyl carbons)*	14.4	14.0	21.5
90-108 *(Anomeric carbons; carbon hydrates)*	3.2	2.4	3.6
108-145 *(Aromatic carbons)*	18.6	10.3	7.4
145-160 *(O-substituted aromatic carbons)*	3.4	1.7	1.4
160-190 *(Carboxylic and ester carbons)*	7.3	8.4	9.1
190-220 *(Carbonyl carbons; ketones and quinones)*	1.8	2.1	1.1

In order to evaluate the influence of light absorption by the different DOM fractions in the photodegradation rates, inner-filter effects were quantified. For this purpose, a wavelength specific screening factor (S_λ) was determined, according to equation 4.1 (Schwarzenbach et al., 2003; Guerard et al., 2009):

$$S_\lambda = \frac{(1 - 10^{-\alpha_\lambda \times b})}{2.303 \times \alpha_\lambda \times b} \quad (4.1)$$

where α_λ (cm^{-1}) is the wavelength specific attenuation coefficient (considered as the absorbance of each DOM fraction, for each wavelength, when the path length is 1 cm – spectra shown in Figure 4.4) and b (cm) is the path length of the irradiated quartz tubes (1.5 cm). The integrated area of a plot of S_λ *versus* λ was determined and divided by the corresponding area when no inner-filter effects occur (S_λ equals 1 for all wavelengths); the obtained value represents the overall screening factor *(S*). Accordingly, *S* (between 290 and 800 nm) due to the presence of humic acids, fulvic acids and XAD4 fraction was 0.993, 0.996 and 0.998, respectively. The determined screening factors allowed to conclude that inner-filter effects due to the absorption of light by DOM is negligible. Thus, the higher incidence of aromatic moieties in humic acids, which results in a higher absorption of UV reactive light (Figure 4.4), cannot explain the decrease in the photodegradation rates observed in the presence of this DOM fraction. In general, the distinct effects caused by the different fractions of humic substances (both enhancing and inhibitory) seem to be exclusively related to the production and/or scavenging of reactive intermediates differently favored by each fraction.

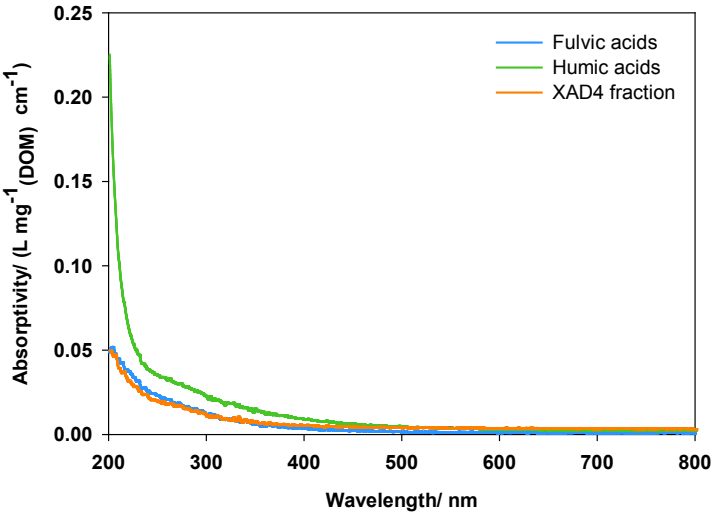

Figure 4.4. UV-Vis spectra of the different dissolved organic matter fractions used in indirect photodegradation studies. The spectra were obtained with a UV-Vis Shimadzu spectrophotometer and a cell with an optical path length of 1 cm was used.

Overall, different fractions of humic substances exerted distinctive effects on the photodegradation rates of benzodiazepines. Nevertheless, half-life times obtained under indirect photodegradation conditions were comparable to those obtained under direct photodegradation, showing that the presence of naturally occurring DOM, at low concentrations (1 mg L^{-1}), is not responsible for a drastic change on the environmental half-life times of these compounds. Interestingly, the photo resistance of alprazolam was not influenced by indirect photodegradation driven by the presence of humic substances, at a reasonable time scale.

4.3.3 Determination of the apparent quantum yield

The average apparent quantum yield (φ_{ave}) of direct photolysis of the studied benzodiazepines was determined using the approach described in chapter 3 (section 3.1.3). Briefly, the determination was based on the overall average of lamp emission intensities in the wavelength range between 290 and 800 nm, by means of the following equation:

$$\phi_{ave} = \frac{C_0 \times k}{\sum I_{\lambda i}^0 \times (1 - 10^{-\varepsilon_{\lambda i} \times b \times C_0})} \quad (4.2)$$

where k is the apparent first order rate constant (s^{-1}), C_0 is the initial concentration of each benzodiazepine in solution (mol L^{-1}), $I_{\lambda i}^0$ is the lamp emission intensity at the wavelength λ_i (Ein L^{-1} s^{-1}), ε is the molar absorptivity of each benzodiazepine at λ_i (L mol^{-1} cm^{-1}) and b is the path length inside the photoreactor (cm) (diameter of the cylindrical quartz tubes, 1.5 cm). Emission lamp spectrum is presented in Figure 3.1a); absorption spectra of each benzodiazepine are presented in Figure 4.5.

The direct photolysis apparent quantum yields are displayed in Table 4.2. Apparent quantum yields of oxazepam, diazepam and alprazolam are in the same order of magnitude (10^{-6}). Consequently, the significantly higher half-life time of alprazolam is explained by poor absorption of solar light and not by a lower photolysis quantum yield. On the other hand, absorption of lorazepam (for wavelengths above 290 nm) is similar to diazepam and oxazepam (or even slightly lower); nevertheless, lorazepam has an apparent quantum yield approximately 20 times higher (in the order of 10^{-5}), justifying the increase on the photodegradation rates of this compound. No literature data is available to compare with the results obtained in this study.

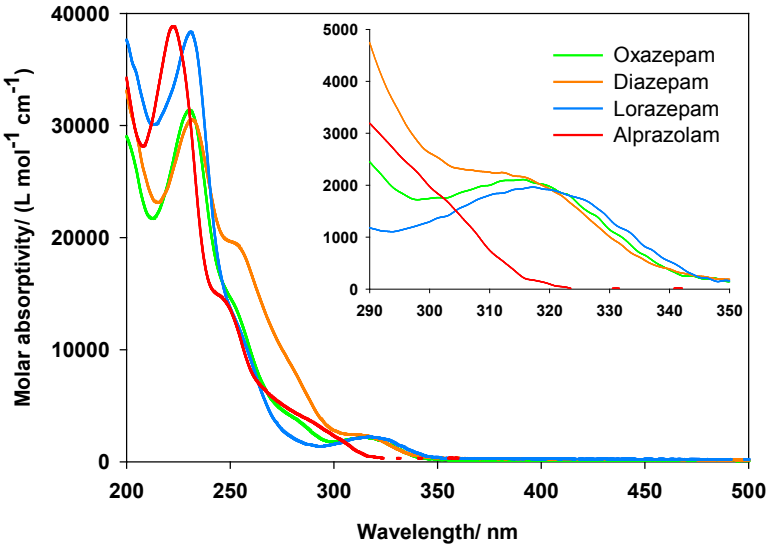

Figure 4.5. UV-Vis spectra of the studied benzodiazepines in aqueous solution. Spectra in the upper right corner are a detailed view of the region between 290 and 350 nm. The spectra were obtained with a UV-Vis Shimadzu spectrophotometer and a cell with an optical path length of 1 cm was used.

4.3.4 Environmental relevance of the results

Half-life times and apparent pseudo-first order rate constants were determined under simulated solar irradiation and are, consequently, strictly related to the specific experimental conditions adopted. Similarly to the procedure adopted in chapter 3, results can be converted into outdoor half-life times, in units equivalent to summer sunny days (SSD) by assuming that the lamp properly simulates sunlight. Considering that the total energy reaching the ground (290 – 400 nm) on a cloudless summer day (15th July, 45ºN latitude) is 7.5×10^{5} J m^{-2} (Vione et al., 2006; Minero et al., 2007), one summer sunny day (a 24 h day/night cycle) corresponds to 3.8 h of irradiation. Results converted to SSD are presented in Table 4.2. Under direct photodegradation conditions, half-life times range between 0.68 ± 0.03 and 228 ± 11 SSD for lorazepam and alprazolam, respectively. While lorazepam might not be considered a persistent compound in the environment, it is expected that the elimination of alprazolam from surface waters by photodegradation processes would take several months. Moreover, diazepam and oxazepam, exhibit half-life times of several days (7.3 ± 0.5 and 3.98 ± 0.04 SSD, respectively), which is also considered environmentally relevant, especially if we take into consideration the continuous introduction of these compounds in the environment by WWTPs. Note that diazepam has a higher environmental half-life time than carbamazepine (4.5 SSD), which is pointed out in the literature as significantly resistant to

photodegradation. The environmental half-life times obtained under indirect photodegradation conditions are consistent with the conclusions taken for direct photodegradation. In general, these results highlight that photodegradation processes might not be efficient elimination pathways to prevent from the accumulation of oxazepam, diazepam and alprazolam in aquatic environments. On the contrary, lorazepam is quickly eliminated by photodegradation.

In order to allow a better understanding of the persistence of benzodiazepines in aquatic environments, further studies involving the role of other constituents of natural waters (such as chloride and nitrates) in the photodegradation processes are mandatory. Furthermore, the environmental relevance of these results is strictly related to the fate of these pharmaceuticals in the water/sediment or soil interface. Once they are introduced into the environment, photodegradation might be more or less relevant depending on the partition of these compounds between aqueous and solid matrices. Literature data regarding sorption of psychiatric pharmaceuticals onto sediments evidenced their complex behavior (Stein et al., 2008), emphasizing that sorption and degradation phenomena should be faced as complementary; only this approach would allow attaining valid conclusions.

4.3.5 Identification of photodegradation products by mass spectrometry

The identification of the photodegradation products was performed by comparing ESI(+)MS spectra of each sample before and after irradiation (examples of ESI-MS spectra used for this purpose are shown in Figures 4.6a) - d)). Subsequently, the molecular ions $[M+H]^+$ and/or $[M+Na]^+$ of possible photodegradation products were selected. The molecular formula and structure of the products were then tentatively assigned based on the fragmentation patterns observed in the ESI-MS^2 spectra (detailed data in Table 4.5). Structures of the studied benzodiazepines and proposed structures for photoproducts are presented in Figure 4.7. A total of 19 benzodiazepines photodegradation products were identified. All photoproducts, with only one exception, have lower molecular weight than the parent compound and photo-induced dimerization was not observed. In general, the majority of the identified compounds is monochlorinated and clearly exhibited (in the ESI-MS spectra) the typical pattern of compounds containing one chlorine atom. Also, the loss of a chlorine radical (-35 Da) or of a HCl molecule (-36 Da) were frequently observed in the respective ESI-MS^2 spectra. Photodegradation products of oxazepam, diazepam and lorazepam mainly resulted from the opening of the diazepinone seven-membered ring followed by a rearrangement into a highly stabilized six-membered ring (as it is the case of OXZ IV-VI, DZP I and LRZ VI), or

even into an aromatic system of 3 adjacent 6 (or 7)-membered rings (DZP III, LRZ II-IV). Differentiation of compounds containing the phenyl ring was possible due to the commonly observed loss of the phenyl group (-78 Da). Oxidation to alcohols and ketones was also frequently observed; the existence of carbonyl and hydroxyl groups was confirmed by the presence of ESI-MS^2 base peaks which correspond to the loss of a CO group (-28 Da) or a H_2O molecule (-18 Da).

Interestingly, and despite from only differing on the presence of one chlorine atom in the phenyl ring, photodegradation of oxazepam and lorazepam resulted in the appearance of distinct photoproducts, showing that the extra ortho-chlorine atom in lorazepam plays a decisive role on photo-induced transformations. The major photodegradation product of lorazepam was LRZ V (M_w 265, $[M+H]^+$ = 266); the emergence of the compound LRZ II (M_w 213, $[M+H]^+$ = 214) should also be highlighted due to its structural similarity with acridine, which is known for its carcinogenic activity (Chiron et al., 2006) (LRZ II corresponds to chloroacridine). In addition, it is important to emphasize that it was possible to differentiate photoproducts with the same molecular weight (OXZ V, $[M+H]^+$ = 257 and LRZ IV, $[M+H]^+$ = 257) based on their different ESI-MS^2 fragmentation patterns.

In the case of alprazolam, the aromatic triazole ring seems to have a protective effect in the seven-membered ring opening and only 2 photodegradation products were identified (ALP I, M_w 297, $[M+H]^+$ = 298 and ALP II, M_w 299, $[M+H]^+$ = 300), also explaining the extremely low photodegradation rate of this compound. These 2 photoproducts also resulted from the opening of the seven-membered ring followed by oxidation to a ketone or alcohol.

In order to further validate the suggested identification of the photoproducts, proposed molecular formulas were confirmed by exact mass measurement and elemental composition determination of the new ions, observed in the ESI-MS spectra after photodegradation. For the determination of the exact mass measurement of the new ions, lock mass was the calculated monoisotopic mass/charge of the non-modified benzodiazepines. The exact calculated monoisotopic masses were compared with observed masses in the ESI(+)MS spectra (detailed results for each photoproduct are displayed in Table 4.6). For a large number of predicted formulas, the errors between the observed and calculated masses are below 9 mDa (OXZ III-V, VII; DZP I, III and LRZ I-VI), confirming with good accuracy the elemental composition of the photoproducts. In the remaining cases, errors between observed and calculated masses did not overcome 28.4 mDa; these higher errors might be, in some cases, explained by the overlap of the isotopic contribution of ^{13}C or ^{37}Cl of other identified products (OXZ VI, DZP IV).

Table 4.5. Ions detected in ESI(+)MS and fragment ions detected in ESI(+)MS^2 used in the identification of the photodegradation products of oxazepam (OXZ), diazepam (DZP), lorazepam (LRZ) and alprazolam (ALP). Relative abundance and respective losses of the relevant fragment ions are also presented. The designation of the photodegradation products is in accordance with Figure 4.7.

Compound	M_w	ESI(+)MS *m/z**	ESI(+)MS^2 *m/z* (relative abundance %, loss)
OXZ	286	287	269 (100, -H_2O); 145 (100, -H_2O and -CO)
OXZ – I	144	145	119 (18, -$CN^{\cdot}$); 101 (100, -$HCONH^{\cdot}$)
OXZ – II	190	191	163 (63, -CO); 145 (100, -CO and -H_2O); 101 (23, -CO, -H_2O and - $HCONH^{\cdot}$)
OXZ – III	231	232	154 (100, -C_6H_6); 126 (2, -C_6H_6 and -CO); 105 (7, -$C_6H_4NH_2Cl$)
OXZ – IV	240	241	214 (2, -HCN); 205 (13, -HCl); 163 (100, -C_6H_6); 138 (10, -C_6H_6 and -HCN)
OXZ – V	256	257	239 (55, -H_2O); 179 (70, -C_6H_6); 151 (100, -C_6H_6 and -CO)
OXZ – VI	257	258	240 (8, -H_2O); 180 (100, -C_6H_6)
OXZ – VII	302	325	308 (3, -$OH^{\cdot}$); 280 (100, -H_2NCHO); 254 (6, -H_2NCHO and -HCN)
DZP	284	285	257 (30, -CO); 228 (42, -H_3CNCO); 222 (73, -CO and -$Cl^{\cdot}$); 193 (100, -H_3CNCO and -$Cl^{\cdot}$)
DZP – I	223	224	179 (8, -HNCHOH); 171 (100, -CH_2CHCN); 153 (41, -CH_2CHCN and -H_2O)
DZP – II	227	228	193 (100, -$Cl^{\cdot}$); 125 (3, $C_6H_6CCH_2^{\cdot}$)
DZP – III	243	244	229 (15, -$CH_3^{\cdot}$); 209 (100, -$Cl^{\cdot}$); 208 (2, -HCl)
DZP – IV	245	246	228 (100, -H_2O); 193 (93, -H_2O and –$Cl^{\cdot}$); 168 (20, -C_6H_6)
LRZ	320	321	303 (47, -H_2O); 275 (100, -H_2O and –CO)
LRZ – I	153	154	126 (100, -CO)
LRZ – II	213	214	179 (100, -$Cl^{\cdot}$); 178 (50, -HCl); 152 (17, -HCN); 151 (28, -H_2CN)
LRZ – III	229	230	195 (100, -$Cl^{\cdot}$); 167 (3, -CO)
LRZ – IV	256	257	230 (75, -HCN); 229 (100, -CO)
LRZ – V	265	266	230 (30, -HCl); 154 (100, -C_6H_6Cl); 139 (13, -$C_6H_6NH_2Cl$)
LRZ – VI	290	291	273 (100, -H_2O); 179 (18, -C_6H_5Cl); 151 (8, -C_6H_5Cl and -CO)
ALP	308	309	281 (100, -N_2); 274 (28, -$Cl^{\cdot}$); 205 (19, -C_6H_5CHN)
ALP – I	297	298	281 (5, -$OH^{\cdot}$); 280 (100, -H_2O)
ALP – II	299	300	282 (28, -H_2O); 247 (100, -H_2O and -$Cl^{\cdot}$); 240 (5, -H_2O and –CHNNH); 206 (91, -H_2O and -C_6H_4); 205 (36, -H_2O and –$C_6H_5^{\cdot}$); 204 (22, -H_2O and C_6H_6)

* *m/z* values of ESI(+)MS ions correspond to $[M+H]^+$, with the exception of photoproduct OXZ - VII which corresponds to$[M+Na]^+$.

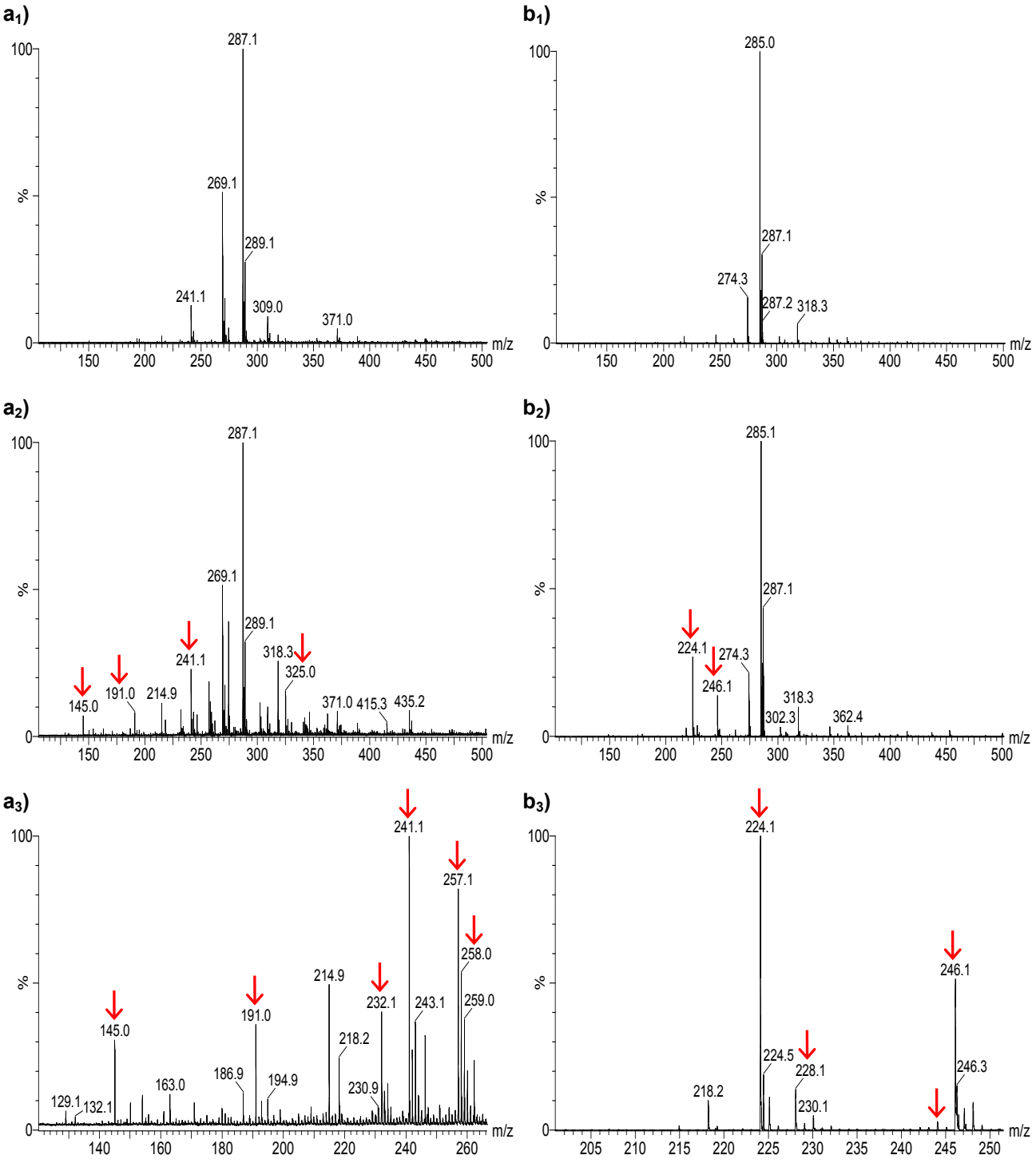

Figure 4.6. ESI-MS spectra of *a)* oxazepam and *b)* diazepam. *1)* Pharmaceutical standard solution *2)* solution irradiated during 24 h, 55 W m^{-2} (290-400 nm), in ultra-pure water; *3)* is a detailed view of spectrum 2*)*. Red arrows draw attention to significant differences in the spectra which correspond to ions of photodegradation products.

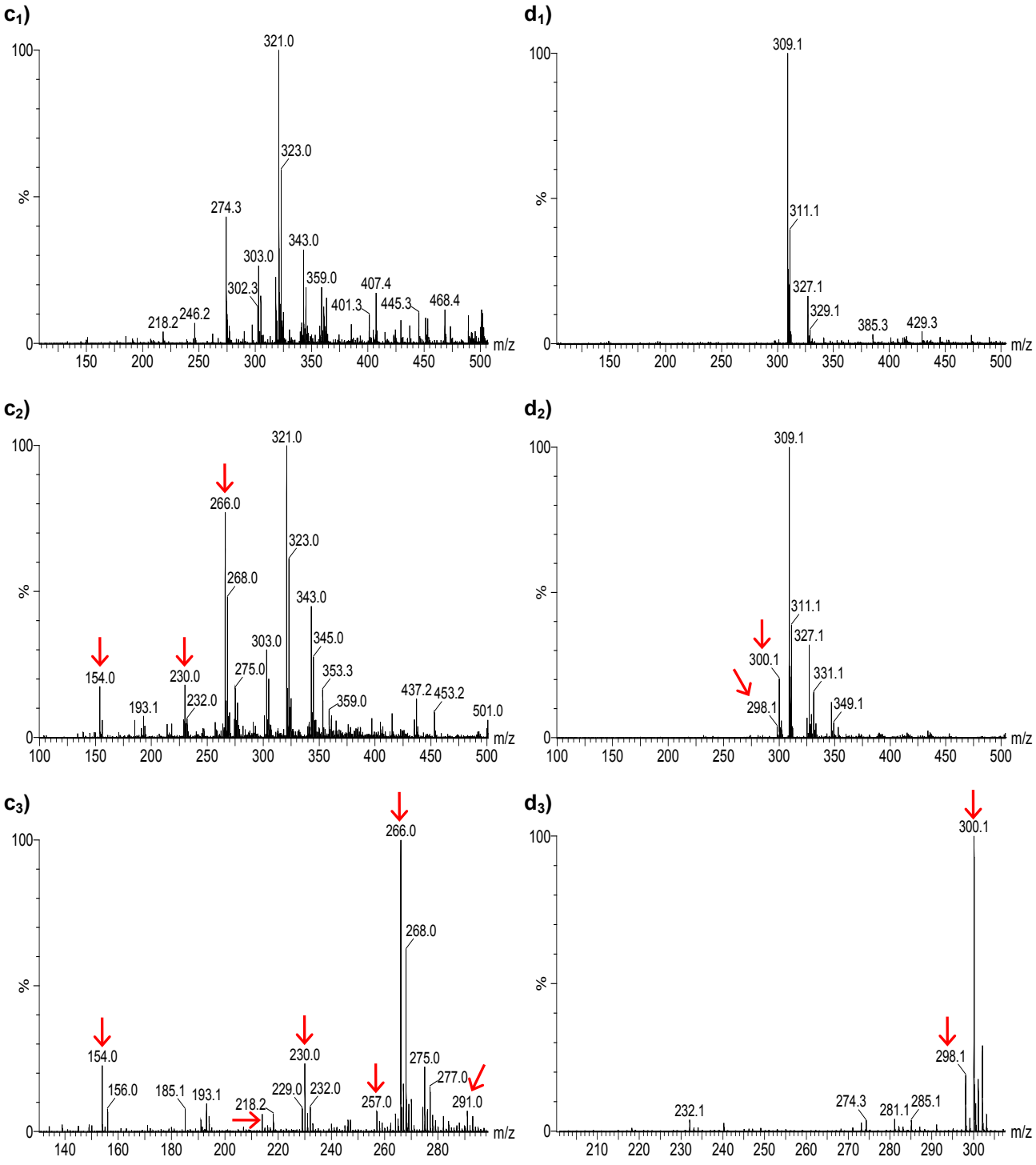

Figure 4.6 (cont.). ESI-MS spectra of *c)* lorazepam and *d)* alprazolam. *1)* Pharmaceutical standard solution *2)* solution irradiated during 24 h, 55 W m^{-2} (290-400 nm), in ultra-pure water; *3)* is a detailed view of spectrum 2*)*. Red arrows draw attention to significant differences in the spectra which correspond to ions of photodegradation products.

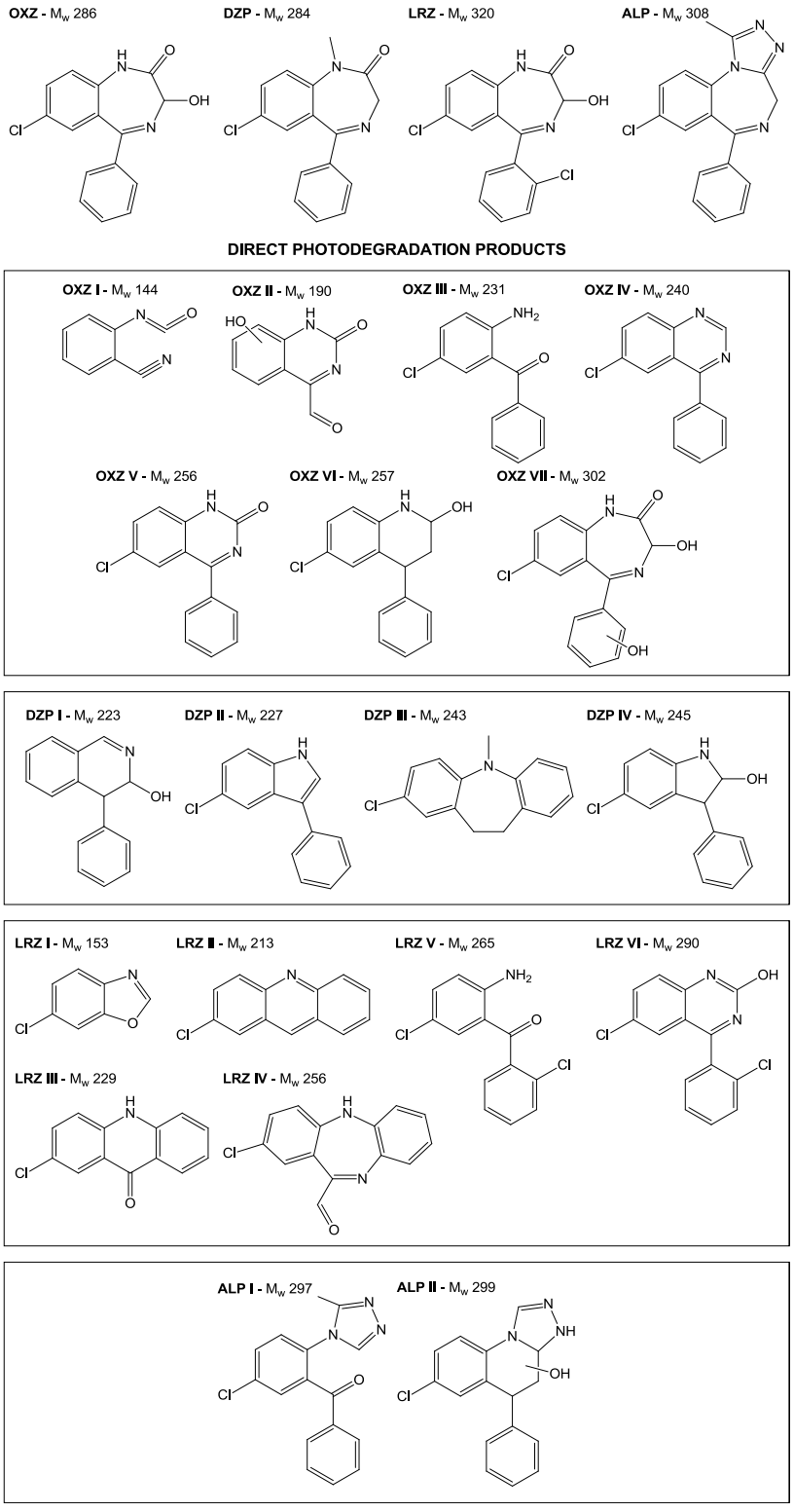

Figure 4.7. Structures of the studied benzodiazepines and proposed structures for photodegradation products. OXZ I – VII, DZP I – IV, LRZ I – VI and ALP I – II correspond to photodegradation products of oxazepam, diazepam, lorazepam and alprazolam, respectively. Molecular weight (M_w) of each compound is also shown.

Table 4.6. Calculated and observed mass, mass error and double bond equivalents (DBE) for the predicted formulas of the identified photodegradation products of oxazepam, diazepam, lorazepam and alprazolam. Predicted formulas correspond to the $[M+H]^+$ ions. The designation of the photodegradation products is in accordance with Figure 4.7.

Compound	Predicted Formula	Calculated mass/ Da	Observed mass/ Da	Error/ mDa	Error/ ppm	DBE
OXZ – I	$C_8H_5N_2O$	145.0402	145.0181	-22.1	-152.3	7.5
OXZ – II	$C_9H_7N_2O_3$	191.0457	191.0219	-23.8	-124.4	7.5
OXZ – III	$C_{13}H_{11}NOCl$	232.0529	232.0544	1.5	6.4	8.5
OXZ – IV	$C_{14}H_{10}N_2Cl$	241.0533	241.0554	2.1	8.9	10.5
OXZ – V	$C_{14}H_{10}N_2OCl$	257.0482	257.0513	3.1	12.2	10.5
OXZ – VI	$C_{15}H_{13}NOCl$	258.0686	258.0415	-27.1*	-104.9	9.5
OXZ – VII	$C_{15}H_{12}N_2O_3Cl$	303.0536	303.0623	8.7	28.6	10.5
DZP – I	$C_{15}H_{14}NO$	224.1075	224.1115	4.0	17.7	9.5
DZP – II	$C_{14}H_{11}NCl$	228.0580	228.0845	26.5	116.2	9.5
DZP – III	$C_{15}H_{15}NCl$	244.0893	244.0859	-3.4	-13.9	8.5
DZP – IV	$C_{14}H_{13}NOCl$	246.0686	246.0963	27.7**	112.7	8.5
LRZ – I	C_7H_5NOCl	154.0060	154.0034	-2.6	-16.7	5.5
LRZ – II	$C_{13}H_9NCl$	214.0424	214.0362	-6.2	-28.7	9.5
LRZ – III	$C_{13}H_9NOCl$	230.0373	230.0356	-1.7	-7.2	9.5
LRZ – IV	$C_{14}H_{10}N_2OCl$	257.0482	257.0468	-1.4	-5.3	10.5
LRZ – V	$C_{13}H_{10}NOCl_2$	266.0139	266.0124	-1.5	-5.8	8.5
LRZ – VI	$C_{14}H_9N_2OCl_2$	291.0092	291.0117	2.5	8.6	10.5
ALP – I	$C_{16}H_{13}N_3OCl$	298.1031	298.0747	28.4	95.2	11.5
ALP – II	$C_{16}H_{15}N_3OCl$	300.0904	300.1137	23.3	77.8	10.5

* Higher mass error due to the isotopic contribution of ^{13}C from the ion at *m/z* 257.

** Higher mass error due to the isotopic contribution of ^{37}Cl from the ion at *m/z* 244.

4.4 CONCLUSIONS

In this study, the relevance of photodegradation processes on the aquatic environmental persistence of benzodiazepines was evaluated for the first time. This work allowed to conclude that photodegradation might not prevent the environmental accumulation of oxazepam, diazepam and alprazolam which were shown to be considerably resistant to direct photodegradation (with half-life times equivalents to 4, 7 and 228 summer sunny days, respectively). On the other hand, lorazepam is susceptible to direct photodegradation, showing a half-life time of less than 1 summer sunny day. The presence of dissolved organic matter resulted in observed half-life times of the same order of magnitude from those obtained under direct photodegradation conditions. Nevertheless, the presence of humic acids was consistent with a decrease in the observed photodegradation rates (which was not due to inner-filter effects), while fulvic acids and XAD-4 fraction caused an enhancement of the photodegradation.

One of the main focuses of this work consisted on the identification of the direct photodegradation products of the studied benzodiazepines, by mass spectrometry. It was possible to identify a total of 19 photodegradation products, the majority of which are reported here for the first time. The identification of the photoproducts is crucial to a more complete understanding of the environmental impact of the presence of benzodiazepines in aquatic systems. The photoproducts originated in the environment should also be considered as relevant environmental pollutants and, optimally, their impact in the environment should also be evaluated. The persistence and/or toxicity of these products might, in some cases, cause a more significant concern than the parent compounds. Thus, the identification of the main photodegradation products, under environmentally relevant conditions, constitutes the first step to allow more comprehensive approaches when assessing scarcely studied pharmaceuticals.

The results presented in this chapter are a valuable tool for the development of further research concerning this topic.

4.5 REFERENCES

Arnold, W.A., McNeill, K., **2007**. Transformation of pharmaceuticals in the environment: photolysis and other abiotic processes. in: Petrovic, M., Barceló, D. (Eds.). *Analysis, Fate and Removal of Pharmaceuticals in the Water Cycle*. Elsevier, Amsterdam, The Netherlands.

Boreen, A.L., Edhlund, B.L., Cotner, J.B., McNeill, K., **2008**. Indirect photodegradation of dissolved free amino acids: the contribution of singlet oxygen and the differential reactivity of DOM from various sources. *Environ. Sci. Technol*. 42, 5492-5498.

Boule, P., Bahnemann, D.W., Robertson, P.K.J. (Eds.), **2005**. *Environmental Photochemistry - Part II*. Springer-Verlag, Berlin.

Cabrera, C.G., de Waisbaum, R.G., Nudelman, N.S., **2005**. Kinetic and mechanistic studies on the hydrolysis and photodegradation of diazepam and alprazolam. *J. Phys. Org. Chem.* 18, 156-161.

Castaneda, B., Ortiz-Cala, W., Gallardo-Cabrera, C., Nudelman, N.S., **2009**. Stability studies of alprazolam tablets: effects of chemical interactions with some excipients in pharmaceutical solid preparations. *J. Phys. Org. Chem.* 22, 807-814.

Chiron, S., Minero, C., Vione, D., **2006**. Photodegradation processes of the antiepileptic drug carbamazepine, relevant to estuarine waters. *Environ. Sci. Technol.* 40, 5977-5983.

Dalrymple, R.e.M., Carfagno, A.K., Sharpless, C.M., **2010**. Correlations between dissolved organic matter optical properties and quantum yields of singlet oxygen and hydrogen peroxide. *Environ. Sci. Technol.* 44, 5824-5829.

Esteves, V.I., **1995**. *Extracção e caracterização de substâncias húmicas de diferentes ambientes aquáticos*, Tese de Doutoramento em Química, Universidade de Aveiro.

Esteves, V.I., Cordeiro, N.M.A., Duarte, A. C., **1995**. Variation on the adsorption efficiency of humic substances from estuarine waters using XAD resins. *Mar. Chem.* 51, 61-66.

Esteves, V.I., Otero, M., Duarte, A.C., **2009**. Comparative characterization of humic substances from the open ocean, estuarine water and fresh water. *Org. Geochem.* 40, 942-950.

Garbin, J.R., Milori, D.M.B.P., Simões, M.L., da Silva, W.T.L., Neto, L.M., **2007**. Influence of humic substances on the photolysis of aqueous pesticide residues. *Chemosphere* 66, 1692-1698.

Guerard, J.J., Miller, P.L., Trouts, T.D., Chin, Y.P., **2009**. The role of fulvic acid composition in the photosensitized degradation of aquatic contaminants. *Aquat. Sci.* 71, 160-169.

Health, N., **2011**. The internet drug index (the top 200 prescriptions for 2009 by number dispensed). http://www.rxlist.com (Accessed January 2011).

International Narcotics Control Board, **2010**. *Psychotropic Substances - Report 2009: Statistics for 2008*. New York.

J.N. Miller, Miller, J.C., **2005**. *Statistics and Chemometrics for Analytical Chemistry*, 5th ed. Prentice Hall, Dorset England.

Leenheer, J.A., Croué, J.-P., **2003**. Peer reviewed: characterizing aquatic dissolved organic matter. *Environ. Sci. Technol.* 37, 18A-26A.

Minero, C., Chiron, S., Falletti, G., Maurino, V., Pelizzetti, E., Ajassa, R., Carlotti, M., Vione, D., **2007**. Photochemincal processes involving nitrite in surface water samples. *Aquat. Sci.* 69, 71-85.

Newman, R.H., **2007**. *Agriculture and Soils*. John Wiley & Sons, Ltd.

OECD, **2008**. *Phototransformation of Chemicals in Water - Direct Photolysis*. Guideline for the Testing of Chemicals nº 316, Paris.

Santos, M.E., Esteves, V.I., Amado, F.L., Duarte, A.C., **1994**. Fractionation and characterization of aquatic organic matter. in: Senesi, N., Miano, T.M. (Eds.). *Humic Substances in the Global Environment and Implications on Human Health*. Elsevier, Amsterdam, pp. 877-882.

Schwarzenbach, R.P., Gschwend, P.M., Imboden, D.M., **2003**. *Environmental Organic Chemistry*. John Wiley & Sons, New Jersey.

Stein, K., Ramil, M., Fink, G., Sander, M., Ternes, T.A., **2008**. Analysis and sorption of psychoactive drugs onto sediment. *Environ. Sci. Technol.* 42, 6415-6423.

Sturini, M., Speltini, A., Maraschi, F., Profumo, A., Pretali, L., Fasani, E., Albini, A., **2010**. Photochemical degradation of marbofloxacin and enrofloxacin in natural waters. *Environ. Sci. Technol.* 44, 4564-4569.

Vione, D., Falletti, G., Maurino, V., Minero, C., Pelizzetti, E., Malandrino, M., Ajassa, R., Olariu, R.-I., Arsene, C., **2006**. Sources and sinks of hydroxyl radicals upon irradiation of natural water samples. *Environ. Sci. Technol.* 40, 3775-3781.

Xia, X., Li, G., Yang, Z., Chen, Y., Huang, G.H., **2009**. Effects of fulvic acid concentration and origin on photodegradation of polycyclic aromatic hydrocarbons in aqueous solution: Importance of active oxygen. *Environ. Pollut.* 157, 1352-1359.

Zafiriou, O.C., Joussot-Dubien, J., Zepp, R.G., Zika, R.G., **1984**. Photochemistry of natural waters. *Environ. Sci. Technol.* 18, 358A-371A.

Zeng, K., Hwang, H.-m., Yuzuri, H., **2002**. Effect of dissolved humic substances on the photochemical degradation rate of 1-aminopyrene and atrazine. *Int. J. Mol. Sci.* 3, 1048-1057.

Zepp, R.G., Schlotzhauer, P.F., Sink, R.M., **1985**. Photosensitized transformations involving electronic energy transfer in natural waters: role of humic substances. *Environ. Sci. Technol.* 19, 74-81.

CHAPTER 5

Quantification of carbamazepine in the environment by ELISA

In this chapter, a previously developed enzyme-linked immunosorbent assay (ELISA) was applied to the quantification of carbamazepine in ground, surface and wastewaters. The performance of the applied ELISA methodology was tested in the presence of high concentrations of sodium chloride and dissolved organic matter. The method was not significantly affected by matrix effects, being adequate for the quantification of carbamazepine in environmental samples, in the range of 0.03 - 10 μg L^{-1}, even without sample pre-treatment. Due to a pH dependent cross-reactivity with cetirizine, an antihistaminic drug, the assay also enabled the quantification of cetirizine in the samples. The developed method was applied to real samples with different matrices, collected in the geographical area of Ria de Aveiro*, an estuarine system located in the North of Portugal. Carbamazepine was detected in all the analyzed wastewater samples and one surface water with concentrations between 0.1 and 0.7 μg L^{-1}. Validation with LC-MS/MS revealed that results obtained by ELISA are 2 to 28% overestimated, which was considered highly satisfactory due to the absence of sample pre-treatments.*

CONTENTS

5.1 CONTEXTUALIZATION

In opposition to other countries located in Occidental and Central Europe (such as Spain, France, Italy and Germany), very limited data is available about the presence of pharmaceuticals in the environment in Portugal. Until now, only a few recent studies were published reporting surface water contamination with endocrine disruptors in the Douro river, the Ria of Aveiro, the Mondego and the Sado estuarine systems (Ribeiro et al., 2009a; Ribeiro et al., 2009b; Ribeiro et al., 2009c; Jonkers et al., 2010). In the specific case of the anti-epileptic carbamazepine, the available literature data is restricted to the works recently published by Madureira et al. (2009, 2010) which reported carbamazepine contamination levels around 178 ng L^{-1} in surface waters (Douro River, Northern Portugal).

As pointed out in previous chapters, carbamazepine has been proposed, in several studies, as an indicator of anthropogenic pollution. In order to suit this purpose at a regional/national scale, apart from carbamazepine´s resistance to degradation and persistence in aquatic environments, local rates of consumption should also be taken into consideration. In Portugal, during the last years, carbamazepine was consistently amongst the most prescribed pharmaceuticals with an estimated consumption of at least 400 000 packages per year (Infarmed, 2008, 2009). Consequently, in the case of Portugal, the assessment of carbamazepine contamination levels might constitute a valuable tool for the identification of relevant focal points of pollution.

In this research, an enzyme-linked immunosorbent assay (ELISA) was applied to the quantification of carbamazepine in ground, surface and wastewaters, collected in Aveiro (Northern Portugal). The ELISA methodology, based on a commercial monoclonal antibody, was previously developed by Bahlmann et al. (2009). Taking into consideration the nature of the collected samples, this study aimed at the evaluation and optimization of the performance of the assay in the presence of high concentrations of organic matter and high salinities in order to develop an ELISA method adequate to perform large environmental screenings. This work also intends to present, for the first time, some results concerning carbamazepine contamination levels in the district of Aveiro. This work was performed in close collaboration with BAM Federal Institute for Materials Research and Testing, Berlin, Germany.

The core concepts involved in the development and application of immunoassays, and ELISA in particular, are presented below. This section also includes a brief characterization of the sampling area.

5.1.1 Immunoassays

The term "immunoassay" comprises a large number of analytical techniques all based on the interaction between an antibody and an antigen (Gault and McClenaghan, 2009). Originally applied to biochemical and clinical analyses (and subsequently to food analyses), immunoassays are now also being used in the quantification of environmental contaminants (Schneider, 2003; Law, 2005; Nunes, 2005). As concluded in chapter 2, the monitorization of pharmaceutically active substances in the environment implies the use of techniques with adequately low limits of quantification (between ng/L and μg/L). In this context, and considering the high sensitivity and specificity that characterize immunoassays, their application to environmental analysis is a growing field of research (Huo et al., 2007; Emon et al., 2008; Shelver et al., 2008; Carvalho et al., 2010). In comparison with reference techniques such as LC-MS/MS or GC-MS/MS, immunoassay methodologies allow the analysis of a large number of samples in a very limited amount of time, revealing their suitability to perform high-throughput environmental screenings (Buchberger, 2007). Moreover, these methods are commonly characterized by operational simplicity and require low-cost equipments; sample pre-treatments (such as pre-concentration procedures, most often coupled to LC and GC methods) are usually not required, which constitutes one of the most striking advantages of immunoassays (Schneider, 2003). Immunoassay techniques are, thus, an inexpensive and time-efficient alternative/complement to chromatographic techniques. The main disadvantage of these methods is their tendency to produce false-positive results (Nunes, 2005; Bahlmann et al., 2011) and, in cases of highly complex matrices (such as wastewater or soil samples) matrix effects might be significant. These points will be object of a closer look through the work presented in this chapter. Additionally, immunoassays are single-analyte techniques and, contrary to chromatographic methods, multi-analyte analyses are only possible by repeated analyses of the same samples.

Immunoassays are not universally accepted as a valuable tool in environmental analysis and, in some cases, examples of application of immunoassays are still scarce. The assay presented here is, to date, the only example reported in literature of an immunoassay method that is being applied to the quantification of a psychiatric pharmaceutical in the environment.

5.1.2 The antibody – main concepts and interaction with the antigen

Antibodies are large glycoproteins of the immune system that belong to the family of immunoglobulins. These biomolecules are intended to specifically bind to an antigen, which is any

substance able to activate an immune response (thus, leading to antibody production) (Harlow and Lane, 1999; Gault and McClenaghan, 2009).

Antibodies contain four polypeptide chains (two identical heavy chains and two identical light chains) and they are grouped in five main classes (IgG, IgM, IgA, IgE and IgD), classified according to the type of the heavy chain. Antibodies are usually described based on the structure of the immunoglobulin G (IgG) which is the most abundant immunoglobulin in the serum (a schematic representation of the IgG is presented in Figure 5.1) (Harlow and Lane, 1999). The light and heavy chains are held together by covalent disulfide bonds and by non-covalent bonds (such as hydrogen bonding). An antibody can be divided in three regions, forming a Y-shaped molecule. The two arms of the Y constitute two identical antigen binding sites (the antibodies are, as a result, bivalent molecules) which are called the Fab domains; the Fab domain is formed by the heavy and light chain variable regions (Harlow and Lane, 1999). The third part of the molecule is constituted by the "tail" of the Y (usually considered the "foot" of the antibody) and is responsible for interacting with other specific molecules of the immune system; it is designated as the Fc domain. The Fc and the Fab domains are connected by a region called "hinge" which allows the Fab domains to perform lateral and rotational movements, conferring to the antibody the ability to undergo conformation-mediated recognition (Harlow and Lane, 1999; Gault and McClenaghan, 2009).

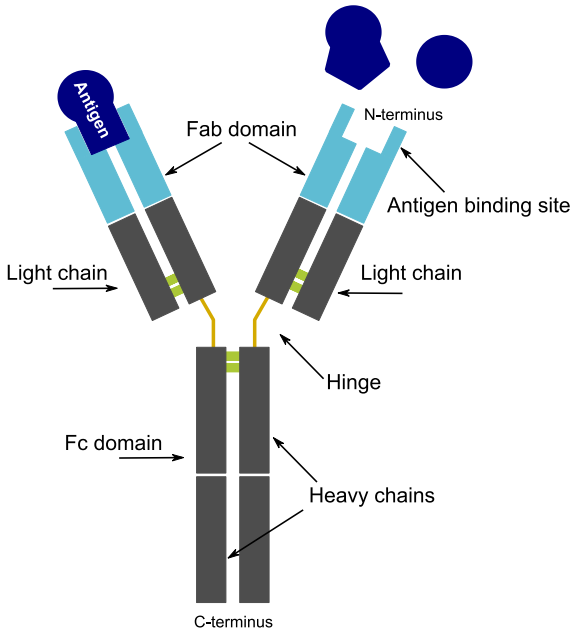

Figure 5.1. Schematic representation of the basic structure of an antibody (immunoglobulin G, IgG), also illustrating the specific interaction between an antibody and an antigen.

Antibodies are produced by the plasma cells of the immune system. The production of antibodies used in immunoanalytical techniques is done by immunization of laboratory animals such as mice, sheep, rabbits or pigs with an immunoconjugate containing the antigen (Nunes, 2005; Gault and McClenaghan, 2009). The presence of the antigen will then induce the production of antibodies by the animals' immune system. After a certain period of time, blood samples are taken, and the antibodies are subsequently purified from the blood serum. However, during the immunologic response, different clones of plasma cells will produce antibodies that will recognize different epitopes (the region of an antigen that is recognized by an antibody and interacts with it) but each clone will only secrete antibodies intended to bind the same epitope. Consequently, the blood serum is a source of polyclonal antibodies (Harlow and Lane, 1999). Monoclonal antibodies, which recognize only a specific epitope, have the main advantage of being highly specific against the selected antigen. To obtain monoclonal antibodies, a culture of a single clone of plasma cells is, however, needed (Gault and McClenaghan, 2009). Due to the complexity involved in the monoclonal antibodies production, there are much more applications based on polyclonal antibodies (Harlow and Lane, 1999).

Frequently, the analytes of interest (antigens) in environmental analysis are not able to elicit an immunological response due to their low molecular weight. Yet they can be recognized and bound by an antibody. So these compounds are called haptens (van Emon, 2007). To overcome this problem, an immunoconjugate is produced by covalently linking the analyte/hapten to a carrier protein (most commonly). When the analyte molecule does not have any functional group that allows the establishment of this covalent link, an extra experimental step is needed: the production of a linkable hapten - a molecule similar to the analyte but modified with a functional group capable of covalently binding to a protein. Consequently, the immune response is not caused by the analyte itself, but by an immunoconjugate that derived from it (Nunes, 2005).

The antibody-antigen interaction is the key to understanding and developing an immunochemical method. It can be accompanied by conformational changes in one, both or none of the molecules. The interaction is mediated by non-covalent bonding which stabilize the antibody-antigen complex and the strength of the interaction is highly dependent on small changes in the antigen structure (Harlow and Lane, 1999).

5.1.3 Enzyme-linked immunosorbent assay (ELISA)

Immunoassays exist in a vast number of formats. "Enzyme immunoassays" is the designation of a group of methodologies that uses an enzyme label (attached either to the antigen or

the antibody) which, in conjunction with a substrate, constitutes the basis of production of the analytical signal. These enzyme labels, designated as "tracers", are most commonly constituted by horseradish peroxidase or alkaline phosphatase (Law, 2005; Nunes, 2005). The classification of the different types of enzyme immunoassays is lacking some clarity; yet, in general, they can be classified into competitive/ non-competitive (whether or not there is competition between the analyte and the tracer for a limited number of antibody binding sites) and heterogeneous/ homogenous (when one of the reagents is immobilized onto a solid support and bound and free reagents should be separated by performing washing steps or the immunoreaction takes place in solution, without any separation of the bound and free reagents, respectively) (van Emon, 2007; Gault and McClenaghan, 2009). One of the most popular formats in environmental analysis is the enzyme-linked immunosorbent assay (ELISA) which is a heterogeneous assay with one of the components (the antigen or the antibody) bound to a solid phase (Law, 2005).

The principle of a direct competitive ELISA (the format optimized in this work) is illustrated in Figure 5.2. In this case, the antigen and a known quantity of tracer directly compete for the antibody binding sites (immobilized onto a solid support, a 96-well microtiter plate). After removing the remaining free reagents in solution (not bound to the antibody) the addition of a substrate results in the development of color (catalyzed by the enzyme tracer). The signal (optical density) is then proportional to the amount of tracer bound to the antibody and inversely proportional to the amount of antigen present in samples/standards.

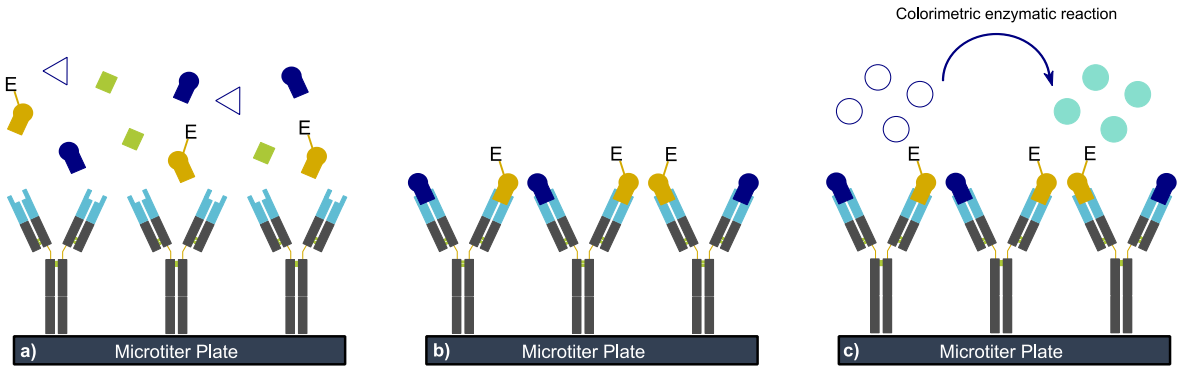

Figure 5.2. Principle of a direct competitive ELISA: a) samples containing the analyte (represented in dark blue) and the tracer (analogue of the analyte and enzyme-labeled, represented in yellow) are added to the microtiter plate, where the antibody is immobilized; b) the analyte and tracer directly compete to occupy the available binding positions of the antibody; c) the addition of a substrate, in the presence of the enzyme-labeled tracer, results in an enzyme catalyzed colorimetric reaction which enables the quantification of the analyte.

5.1.4 ELISA calibration curve

The use of appropriate calibration models is fundamental to the overall quality of an immunoassay, as for all analytical methods. In ELISA, the relationship between the response and the analyte concentration is characterized by non-linear relationships. As the calibration curve fitting is non-linear, choosing an appropriate fitting model demands special attention. Additionally, the response can be directly or inversely proportional to the analyte concentration, depending on the format of the assays (Findlay and Dillard, 2007).

Typically, ELISA calibration curves are sigmoidal with a lower and an upper asymptote which correspond to infinite and zero analyte concentrations, respectively. The 4-parameter logistic (4PL) model is the most commonly used for fitting ELISA calibration curves (equation 5.1).

$$y = D + \frac{A - D}{1 + \left(\frac{x}{C}\right)^B} \quad (5.1)$$

where, *y* is the response, *D* is the response at infinite analyte concentration, *A* is the response at zero analyte concentration, *x* is the analyte concentration, *C* is the abscissa of the inflection point (the test midpoint) and *B* is a slope parameter (which defines the steepness of the fitting). The curve is symmetric around the test midpoint. The 4PL model is considered to provide the best fit across the entire calibration range. Other models, such as linear, exponential and quadratic are also referred to in literature. Nevertheless, they are characterized by a reasonable fitting only in reduced areas and the fitting is most often unsatisfactory at higher and lower concentration ranges (Findlay and Dillard, 2007).

The quality of the calibration curve also reflects the adequacy of the assay design and plate layout. Some of the main aspects to take into account when performing ELISA are (Ekins, 1981; Findlay and Dillard, 2007):

- Use between five and eight calibrators to allow a proper curve fitting but, at the same time, avoid reducing the number of samples that can be analyzed in the same experiment;
- Distribute the calibrator concentrations logarithmically and analyze each of them, ideally, in triplicate;
- Include calibrators with concentrations that are outside the working range;
- Assign the calibrators and samples as randomly as possible to the microtiter plate wells, in order to avoid systematic errors due to position in plate and drift effects.

In the fitting process, it is also important to consider that the variance of the response is a function of the response; the variance is, thus, heteroscedastic. Consequently, weighted calibration curves, where response with higher variation have a minor relative importance in the fitting when compared to points with lower variation, should be applied.

5.1.5 Cross-reactivity

As previously explained, the region of an antigen that is recognized by an antibody and interacts with it is the epitope. The epitope is not a property of any compound and its definition only makes sense when referred to the interaction with a specific antibody. Epitopes are very often relatively small regions of the antigen and, thus, it is not uncommon that antibodies interact with other compounds that have structures related to the epitope (or sometimes detect spatial arrangements similar to the one of the epitope). This recognition of compounds other than the antigen is known as cross-reactivity (Harlow and Lane, 1999).

The cross-reactivity is the basis of the specificity of an immunoassay. For this reason, the validation of an immunoassay should always comprise an evaluation of the cross-reactivity levels with potential cross-reactants. The potential interfering compounds should be selected amongst chemicals that show structural similarities with the antigen, such as metabolites and degradation products. However, for obvious reasons, it is impossible to effectively select and test all the compounds that might possibly interact with the antibody, resulting in ignoring possible cross-reactants that are not obviously structurally correlated with the antigen (Law, 2005; Bahlmann et al., 2011).

To evaluate the cross-reactivity towards a battery of compounds, these should be assayed as if they were the analyte of interest. The calibration curve obtained for each compound is then compared with the curve of the analyte (Law, 2005). Most commonly, the comparison of both curves is done by using the 50% displacement method: the cross-reactivity is expressed as the relative dose, in percentage, required to cause a 50% displacement of the maximum tracer binding (50% maximum inhibition concentration, IC_{50}), as follows (Law, 2005):

$$\text{Cross reactivity} = \frac{\text{IC}_{50\ \text{analyte}}}{\text{IC}_{50\ \text{competitor}}} \times 100\% \quad (5.2)$$

Note that the IC_{50}, that is equivalent (but not equal) to the test midpoind (the ELISA curve inflection point), is also very often used to express the sensitivity of the assays (Nunes, 2005).

When interpreting the results, cross-reactivities should be expressed in terms of molar concentrations. This is particularly relevant when the molecular weights of the analyte and the competitor significantly differ (Law, 2005).

One of the main disadvantages of immunoassays is a direct consequence of the cross-reactivity: the assays might have a tendency to produce false-positive results, due to the recognition of compounds that are not the analyte. However, for some purposes, it is also possible to take advantage of the cross-reactivity as it might enable the determination of a whole class of compounds or multi-analyte approaches (Schneider et al., 1992; Harlow and Lane, 1999).

5.1.6 Assay precision – the precision profile and the working range

The determination of the precision profile is considered to be the best method to evaluate the precision both within and between ELISA experiments (Law, 2005). This method was first developed by Ekins (1981), who defined the precision profile as "a graphical representation of the random error in the analyte measurement at each value of the analyte concentration". Thus, it represents the relationship between the concentration of the analyte and its measured precision (O'Malley, 2008).

As concluded in a previous section, the calibration curve of ELISA is not linear and, consequently, the error of the concentration is not a linear function of the response error. Both the error of the response and the slope of the calibration curve vary from point to point (Ekins, 1981; Law, 2005). So, as proposed by Ekins, at a certain point, the error of the concentration ($error_x$), is given by:

$$\text{error}_x = \frac{\sigma_y}{\text{slope of the calibration funtion}} \quad (5.3)$$

where σ_y is the standard deviation of the response for replicate measurements. The determination of this quotient for a number of points along the ELISA calibration curve allows building the precision profile. The slope of the calibration curve, at a certain concentration, is given by the first derivative of the calibration curve (Law, 2005). One possible representation of the precision profile is in terms of relative error of concentration (coefficient of variation, C.V.). Therefore, at each point, the precision profile is given by:

$$\text{C.V.} = \frac{\sigma_y}{\text{slope}} \times \frac{1}{x} \times 100\% \quad (5.4)$$

with *x* representing the concentration of the analyte. When the data is fitted to a 4PL model (equation 5.1), the precision profile can be determined as follows:

$$\mathrm{C.V.} = -\sigma_y \times \frac{1}{\mathrm{B(D-A)}} \times \left[2 + \left(\frac{x}{\mathrm{C}}\right)^{\mathrm{B}} + \left(\frac{\mathrm{C}}{x}\right)^{\mathrm{B}}\right] \times 100\% \quad (5.5)$$

The precision profile can be obtained by analyzing a series of calibrators, including at least one calibrator outside the expected upper and lower limits of the quantitation range. It is also mandatory to perform a reasonable number of replicates of the calibrators analysis, to allow obtaining a reliable estimate of the concentration error (Law, 2005). The precision profile is also considered the best method to determine the quantitation or working range that is defined as the highest and lowest concentrations which can be determined with an acceptable degree of precision (Ekins, 1981; Law, 2005).

5.1.7 Characterization of the sampling area

Ground, surface and wastewaters were collected in and around Aveiro (NW Portugal). Aveiro is a small city, surrounded by the *Ria de Aveiro*, a coastal lagoon adjacent to the Atlantic Ocean. It is one of the most important estuarine systems of Portugal, with approximately 45 km^2 of surface area, characterized by the existence of a large and complex network of branches and narrow canals which cross both urban and rural areas.

Domestic wastewaters from Aveiro and the surrounding area are processed at three main wastewater treatment plants (WWTPs). The treated wastewater is discharged into the Atlantic Ocean, at a distance of 3.2 km from the coast, at approximately 17 m depth. The effluent from this outfall ("São Jacinto") corresponds to approximately 28 million m^3 of treated effluent per year (SimRia, 2010). North and south WWTPs are the two main treatment plant facilities, responsible for receiving wastewaters of 272 000 and 159 000 inhabitant equivalents, respectively. In these WWTPs, wastewaters are currently subjected to a pre-treatment, primary decantation, biological treatment and secondary decantation before effluent rejection. The described wastewater treatment system is the result of significant improvements during the last decade, which are still taking place (SimRia, 2010). Before these enhancements, the system was organized in a larger number of smaller WWTPs, from which at least one (located in the center of Aveiro) was directly discharging the effluent into the *Ria de Aveiro* (Sousa et al., 2010).

5.2 MATERIALS AND METHODS

5.2.1 Chemicals

The polyclonal antibody against mouse (IgG F(c) domain, from goat, lot 20185) and the anti-carbamazepine monoclonal antibody (mouse IgG1, clone B3212M, lot 5 K32007) were purchased from Acris Antibodies (Germany) and BIODESIGN International (Meridian Life Science Inc., USA), respectively. The tracer was previously produced and characterized as described in Bahlmann et al. (2009). 3,3',5,5'-Tetramethylbenzidine (TMB, puriss.) and tetrabutylammonium borohydride (TBABH, >97%) were purchased from Fluka. Sodium phosphate dibasic dihydrate (>99%), sodium phosphate monobasic dihydrate (>99%), potassium sorbate (>99%), potassium dihydrogen citrate (>99%), hydrogen peroxide (30%) and Tween^{TM} 20 were also from Fluka. Ethylenediaminetetraacetic acid disodium salt dihydrate (p.a.), and sodium chloride (99.5%) were from Panreac (Spain). Dimethylacetamide (DMA), tris(hydroxymethyl) aminomethane (TRIS, p.a.) and bovine serum albumin (BSA, for electrophoresis, 98%) were purchased from Sigma. Commercial humic acids (technical) were also obtained from Sigma. Glycine (99.8%) was purchased from VWR Prolabo and sodium hydroxide (>98.0%) was obtained from José Manuel Gomes dos Santos (Lisbon, Portugal). Carbamazepine (99%), cetirizine dihydrochloride (>98.0%), epoxy-carbamazepine (98%) were purchased from Sigma. 2-OH-carbamazepine (98%), 10,11-dihydro-10-OH-carbamazepine (10-OH-carbamazepine, 98%), 10,11-dihydro-*trans*-10,11-dihydroxy-carbamazepine (DiOH-carbamazepine, 98%) and 3-OH-carbamazepine (98%) were supplied by Toronto Research Chemicals (Toronto, Canada).

Ultra-pure water, used in the preparation of all the solutions, was obtained using a Millipore water purification system (Milli-Q plus 185).

5.2.2 Materials

Transparent 96 flat-bottom well microtiter plates with high binding capacity (MaxiSorp^{TM}) were purchased from Nunc (Thermo Scientific). Membrane filters (pore size 0.22 μm, Millex-GV) and paper filters (pore size 0.45 μm) were purchased from Millipore. A Titramax 100 plate shaker (Heidolph, Germany) and an automatic 8-channel plate washer (Biochrom, ASYS Atlantis) were also used. Optical density was read using a microplate spectrophotometer (Biochrom, ASYS UVM340).

The LC-MS/MS experiments were performed on an Agilent 1100 LC system consisting of a degasser, binary pump, autosampler and column heater. The outlet of the column was coupled to an API 4000 mass spectrometer from Applied Biosystems. A Turbo V^{TM} ion source was used in electrospray positive mode.

5.2.3 Sample collection and preparation

Water samples (250 mL) were collected in and around Aveiro (NW Portugal), in sites geographically distributed as shown in Figure 5.3.

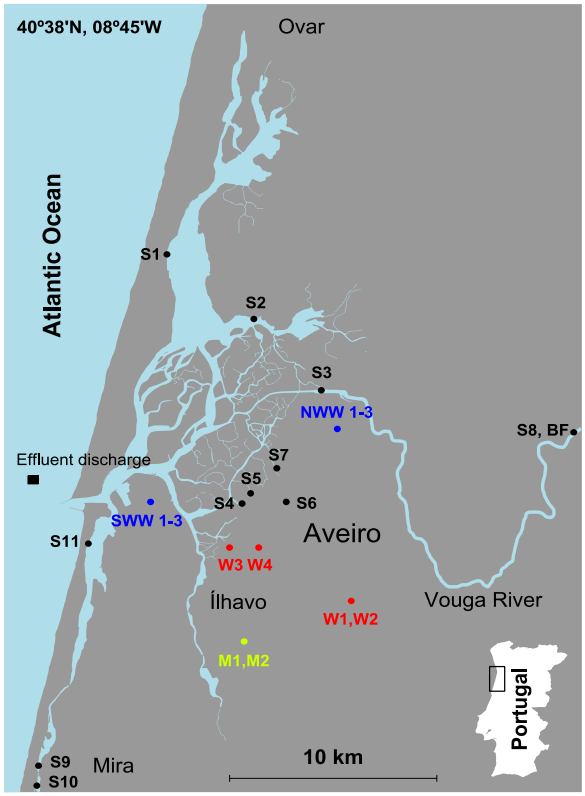

Figure 5.3. Schematic representation of the sampling region around *Ria de Aveiro*. Circles represent collection sites of ground water samples from wells (W1-W4), riverine bank filtration systems (BF) and mines (M1 and M2); surface water samples from *Ria de Aveiro* (S1-S11) and wastewater samples from the two main WWTPs of Aveiro (North, NWW1-3 and South, SWW1-3). The dark square represents the location of the final effluent discharge of the two WWTPs.

Ground water samples were collected from 4 wells (W1 – W4), 2 mines (M1, M2) and 1 riverine bank filtration system on Vouga river (BF), mainly located in rural areas, which are currently used for the water supplies of the municipal drinking water system. Also, 11 surface

water samples (S1 – S11) were collected from North to South of *Ria de Aveiro*, including samples from rural areas (S2, S3, S8), urban areas (S4-S7) and touristic coastal areas (S1, S9-S11). Wastewater samples were collected from the two main WWTPs of Aveiro ("North" and "South") and three collection points were selected: after primary decantation, after secondary biological treatment and after secondary decantation (corresponding to the final treated effluent). Samples were collected during spring (between April and May 2010). All the samples were filtered through 0.45 μm nitrocellulose membrane filters (Millipore), immediately after collection, and stored at -20 ºC until analysis. Samples were not subjected to any other cleaning procedure, extraction or enrichment process.

5.2.4 Immunoassay procedure

The ELISA procedure applied in this study was previously developed; detailed information about tracer characterization and assay development is presented in Bahlmann et al. (2009).

High binding capacity microtiter plates were first coated with polyclonal antibody (anti-mouse IgG, 1 mg L^{-1}, 200 μL per well), covered with Parafilm® to prevent evaporation and incubated overnight (approximately 16 to 18 hours) on a plate shaker at 750 rpm. The antibody dilution was prepared in PBS (10 mM sodium dihydrogen phosphate, 70 mM sodium hydrogen phosphate and 145 mM sodium chloride, pH 7.6). The plates were then washed thrice with PBS 0.05% Tween^{TM} 20 using an 8-channel plate washer. Subsequently, monoclonal antibody against carbamazepine, also diluted in PBS (12.9 μg L^{-1}, 200 μL per well), was incubated for 1 h. After another three-cycle washing procedure, the tracer solution was added to the plates (177 pmol L^{-1} in sample buffer, 50 μL per well). Sample buffer consisted of 1 M glycine, 3 M sodium chloride and 2% (*w/v*) of ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), pH 10.5. Variations in the sample buffer composition, introduced to study its influence on the performance of the assays, are detailed in Sections 5.2.6 and 5.2.7. Then, carbamazepine standard solutions and samples (150 μL per well) were added; the plates were incubated for 30 min and submitted to a final three-cycle washing step. Afterwards, substrate solution (200 μL per well) was pipetted and incubated for another 30 min. The substrate solution consisted of 540 μL of a TMB-based solution (41 mM TMB, 8 mM TBABH in DMA, prepared under nitrogen atmosphere) in 21.5 mL of substrate buffer (200 mM citric acid, 0.01% sorbic acid potassium salt and 3 mM hydrogen peroxide, pH 4.0) and was freshly prepared for each plate. Finally, the reaction was stopped by adding sulfuric acid (1 M, 100 μL per well) and the optical density was read (at 450 nm and

referenced to 650 nm) on a microplate spectrophotometer. Data was analyzed using SoftMax® Pro Software (version 5.3, Molecular Devices).

5.2.5 ELISA calibration curve and precision profile

To obtain ELISA calibration curves, a 100 mg L^{-1} stock solution of carbamazepine was prepared in methanol. The calibrators were subsequently prepared by diluting the stock solution in ultra-pure water, ensuring that the concentration of methanol did not exceed 1% (*v/v*). For quantification purposes, 8 standard solutions, logarithmically distributed, were used with concentrations ranging from 0 to 100 μg L^{-1}. To determine the quantitation range, 16 standard solutions were assayed (with 6 replicates each). Subsequently, the relative error of the carbamazepine concentration was calculated to obtain the precision profile of the assay as described in section 5.1.6. A maximum allowable relative error of 30% for the quantification of carbamazepine in a sample was established as the criterion to define the quantitation range of the assays. The inverse of the square of the standard deviation of replicates was used to allocate the weight of a calibrator in the fitting process. Standard solutions were randomly distributed over the 96 wells of the microtiter plates to level out the influence of possible systematic errors that might arise from signal drifts across the plate.

5.2.6 Determination of cross-reactivity with cetirizine

The antihistaminic pharmaceutical cetirizine was previously identified as an important cross-reactant of this assay (Bahlmann et al., 2011). The study of the immunoassay selectivity for carbamazepine relative to cetirizine was performed by establishing an ELISA curve for cetirizine, using seven standard solutions of this compound with concentrations ranging from 0.03 to 100 μg L^{-1}, and for carbamazepine itself.

The molar cross-reactivity of cetirizine towards carbamazepine was calculated considering the ratio of the molar concentrations of the two compounds at the test midpoint of the correspondent calibration curves, expressed in percentage relatively to the carbamazepine midpoint, according to:

$$\mathrm{CR} = \frac{\mathrm{IC}_{50}\ (\mathrm{Carbamazepine}\ )}{\mathrm{IC}_{50}\ (\mathrm{Cetirizine}\ )} \times 100\% \quad (5.6)$$

In order to evaluate the pH effect on the affinity of the assay towards cetirizine, the cross-reactivity tests were performed at 3 pH values: 4.5, 7.6 and 10.5. A sample buffer, added at the same time of sample incubation, was used to control the pH and ionic strength of the assays. Tested sample buffers have the following compositions: 1 M citrate and 3 M NaCl (pH 4.5); 1 M TRIS, 2% (*w/v*) EDTA and 3 M NaCl (pH 7.6) and 1 M glycine, 2% (*w/v*) EDTA and 3 M NaCl (pH 10.5).

5.2.7 Salinity and dissolved organic matter effects

To study matrix effects on the performance of the assays, the influence of dissolved organic matter (particularly relevant for the analysis of wastewater samples) and sodium chloride concentration (relevant for surface waters collected in *Ria de Aveiro*) were evaluated.

Considering dissolved organic matter effects, ELISA calibration curves were obtained for carbamazepine standard solutions containing 0.1, 1.0 and 10.0 mg L^{-1} of commercial humic acids. Similarly, for salinity effects, carbamazepine standard solutions with 1.0, 10.0 and 30.0 g L^{-1} of sodium chloride were assayed. In both cases, results were compared with those obtained with carbamazepine standard solutions in the absence of humic acids or sodium chloride. The effect of pH and composition of the sample buffers on these experiments was also tested. Organic matter and salinity tests were performed using sample buffers with pH 7.6 and 10.5 with and without the addition of BSA 1% (*w/v*). After selecting the optimal conditions of the assay, some additional tests to check for matrix effects, using real samples, were carried out. Wastewater samples were spiked with 0.5, 1.0 and 1.5 μg L^{-1} of carbamazepine and analyzed by ELISA.

5.2.8 LC-MS/MS method

Samples were analyzed by LC-MS/MS, established as a reference methodology, in order to validate results obtained by ELISA. The LC-MS/MS methodology was adapted from Bahlmann et al. (2009). The instrument's sensitivity was high enough to allow injection of samples without performing any SPE-enrichment procedure. It was previously concluded that results obtained by SPE-LC-MS/MS and LC-MS/MS are not significantly different (Bahlmann et al., 2009).

The chromatographic separation was carried out on a Purospher RP-C18 column (250×3 mm; 5 μm; VDS Optilab, Berlin, Germany) and a guard column was used (10×3 mm). The temperature of the column oven was kept at 40 ºC. The flow rate was maintained at 400 μL min^{-1}

and the sample injection volume was 20 μL. A binary gradient consisting of 5×10^{-3} M ammonium acetate in water (A) and methanol (B) was used. The separation started with 40% of B, maintained at isocratic conditions for 3 min, linearly increased to 95% of B for the period of 15 min, kept at 95% of B during 10 min, linearly decreased to 40% of B within 1 min and finally these conditions were maintained during 10 min, with a total running time of 39 min.

The mass spectrometry detector was used with the following parameters: collision gas 41 kPa, curtain gas 172 kPa, ion source gas 1 and 2 at 345 kPa and 414 kPa, respectively. The source temperature was 400 ºC, entrance potential was set to 10 V and declustering potential set to 60 V. The ion spray voltage was adjusted to 4.5 kV. For the quantification and identification of carbamazepine in the samples, multiple-reaction monitoring mode (MRM) was chosen, monitoring the transitions *m/z* 237→194 and 237→179 for carbamazepine and *m/z* 239→196 and 239→180 for carbamazepine-d_2, used as internal standard. The quantification of some metabolites of carbamazepine was also simultaneously performed, by using the same LC-MS/MS method. The analyzed metabolites (and chosen MRM transitions) are: 10-OH-carbamazepine (*m/z* 237→194 and 255→237), 2-OH-carbamazepine and 3-OH-carbamazepine (*m/z* 253→210 and 253→208), di-OH-carbamazepine (*m/z* 253→180 and 271→253) and epoxy-carbamazepine (*m/z* 253→180 and 253→236).

Acquisition and analysis of data were performed using the Analyst™ 1.4.1 software (Applied Biosystems).

5.3 RESULTS AND DISCUSSION

5.3.1 Cross-reactivity towards cetirizine – pH effect

The specificity of the monoclonal antibody, considering 14 potential cross-reactants, has been studied before (cross-reactivities of some selected compounds are presented in Table 5.1) (Bahlmann et al., 2009). Only epoxy-carbamazepine and 2-hydroxy-carbamazepine, with molar cross-reactivities, at pH 7.6, of 73 ± 9% and 14 ± 1%, respectively, were considered to potentially affect the quantification of carbamazepine in environmental samples; these 2 metabolites are also known to occur in the environment, although usually in lower concentrations than carbamazepine (Miao and Metcalfe, 2003; Miao et al., 2005; Leclercq et al., 2009). However, further investigation performed by the same authors resulted in the discovery of an important cross-reactant that has no obvious structural correlation with carbamazepine; this cross-reactant is cetirizine (Figure 5.4), an

antihistaminic pharmaceutical (Bahlmann et al., 2011). Cetirizine was also recently found in the environment (Kosonen and Kronberg, 2009; Huerta-Fontela et al., 2010; Loos et al., 2010); consequently, it is important to investigate the possible influence of this compound in the analysis of carbamazepine in the collected samples.

Table 5.1. Mean values (± standard deviations) of molar cross-reactivities of the main carbamazepine metabolites at pH 4.5, 7.6 and 10.5. Brackets represent the number of experiments used in the calculation. Data published in Bahlmann et al. (2009, 2011).

Compound	pH 4.5	pH 7.6	pH 10.5
Epoxy-CBZ	67 ± 6% (5)	73 ± 9% (6)	68 ± 4% (4)
2-OH-CBZ	14 ± 1% (4)	14 ± 1% (5)	0.81 ± 0.15% (3)
3-OH-CBZ	2.2 ± 0.7% (4)	1.7 ± 0.1% (4)	0.14 ± 0.01% (3)
10-OH-CBZ	0.56 ± 0.01% (2)	0.47 ± 0.16% (3)	0.50 ± 0.07% (2)
DiOH-CBZ	0.010% (1)	0.014 ± 0.01% (3)	0.009% (1)

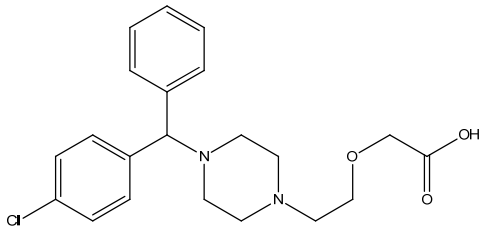

Figure 5.4. Structure of the antihistaminic pharmaceutical cetirizine.

The affinity of the monoclonal antibody towards cetirizine is highly dependent on the pH during the interaction step. This pH dependence is clearly demonstrated in Figure 5.5, where carbamazepine and cetirizine calibration curves at *a)* pH 4.5, *b)* pH 7.6 and *c)* pH 10.5 are presented. The affinity of the antibody towards cetirizine greatly increased from high to low pH, with experimental molar cross-reactivities of 15, 153 and 340% at pH 10.5, 7.6 and 4.5, respectively. These results highlight that carbamazepine concentrations determined at a given pH might be significantly overestimated by the presence of cetirizine in the samples. However, and interestingly, this brings the possibility of the simultaneous determination of cetirizine and carbamazepine with the same ELISA methodology (see Section 5.3.5). To better clarify this matter, all the collected samples were analyzed at pH 4.5 and 10.5. This preliminary study showed that the observed concentrations of carbamazepine at pH 4.5 were up to 180% higher than the ones

determined at pH 10.5. This observation pointed out to the presence of cetirizine in the samples and was crucial to choose the optimal pH conditions for the quantification of carbamazepine in environmental samples with minor influence of cetirizine.

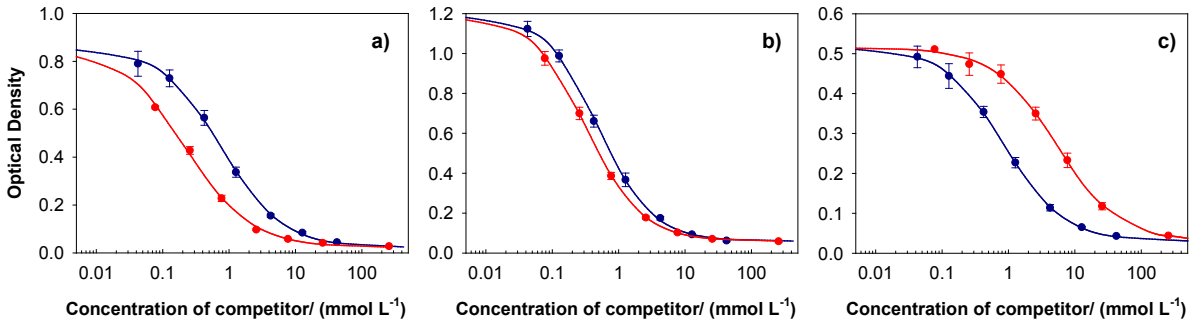

Figure 5.5. ELISA calibration curves for carbamazepine (in blue) and cetirizine dihydrochloride (in red) at *a)* pH 4.5, *b)* pH 7.6 and *c)* 10.5, 3 replicates per calibrator; error bars correspond to standard deviation.

5.3.2 Salinity and dissolved organic matter effects

Matrix effects are a major issue on the analysis of environmental samples, due to their common high complexity. In the present study, the chemical characteristics of ground, surface and wastewaters vastly differ; the presence of sodium chloride and dissolved organic matter were selected as the most relevant interferences to take into consideration.

Surface water samples collected from the Aveiro Lagoon (*Ria de Aveiro*) have sodium chloride concentrations up to 30.0 g L^{-1}. To level out the high concentration of salt, sample buffers (1 M glycine, pH 10.5 or 1 M TRIS, pH 7.6) with 3 M of sodium chloride and 2% EDTA (*w/v*) were tested and the effect of high sodium chloride concentrations in the sample was assessed (Figure 5.6). The sample buffer at pH 7.6 successfully eliminated possible matrix effects caused by sodium chloride. In the case of the sample buffer with pH 10.5, the observed ELISA signal slightly decreased with the increase of the sodium chloride concentration. This shift seems to be more significant for carbamazepine concentrations below 0.1 µg L^{-1}. Overall, sample buffer at pH 7.6 is more effective in the decrease of salinity related matrix effects; however, both sample buffers proved to give satisfactory results for carbamazepine concentrations in a broad range around the test midpoint.

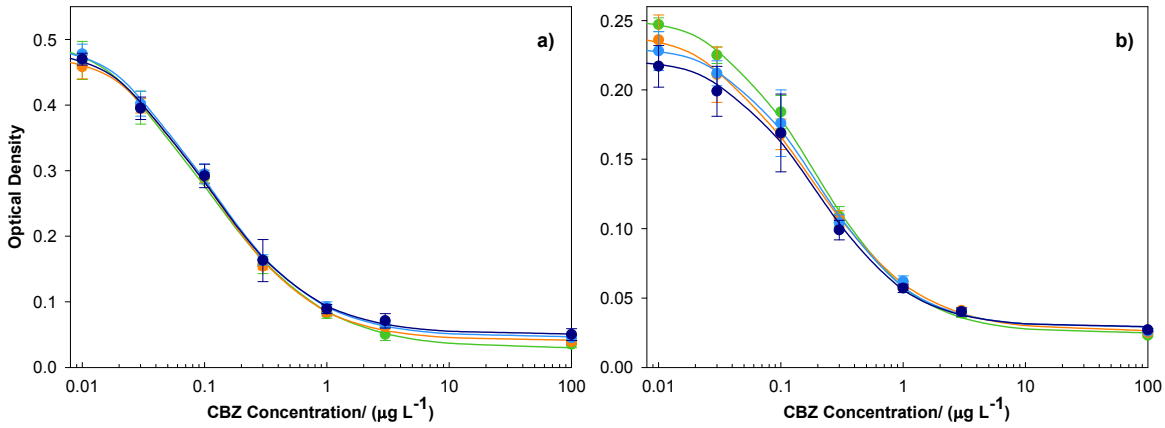

Figure 5.6. Evaluation of salinity effects on the ELISA calibration curves, at pH *a)* 7.6 and *b)* 10.5. Carbamazepine calibrators in the absence of sodium chloride and in the presence of 1.0, 10.0 and 30.0 g L^{-1} of sodium chloride are represented in green, orange, blue and dark blue, respectively.

To evaluate the effect of dissolved organic matter on the performance of the assay, calibrators containing 0.1, 1.0 and 10.0 mg L^{-1} of commercial humic acids were assayed and compared with calibration curves obtained without humic acids. BSA (bovine serum albumin) is a potential agent to bind organic matter (Kalman and Turner, 2007) and thus it was tested if its addition led to any beneficial effect on the assay's performance. The obtained results are shown in Figure 5.7. Only for the highest concentrations of humic acids there was a slight decrease of the observed optical density. This effect was only noticeable at carbamazepine concentrations below 0.1 µg L^{-1}. The presence of BSA did not contribute to a noteworthy decrease of the matrix effects. On the contrary, the addition of BSA increased the observed test midpoint and thus decreased the sensitivity of the test. Furthermore, it was difficult to pipet BSA solutions of the indicated concentration due to the formation of air bubbles. Taking these facts into account, the usage of BSA in the analysis of environmental samples was not considered.

Relative to organic matter effects, it is important to underline that these experiments only gave a first indication on the performance of the assay under the chosen conditions. The obtained results might not be representative for the effect of dissolved organic matter in real samples, which are expected to have a more complex composition than the commercial humic acids tested. Hence, wastewater samples with the highest concentration of organic matter were selected and spiked with 0.5, 1.0 and 1.5 µg L^{-1} of carbamazepine. A graphical representation of the carbamazepine concentration in spiked samples, determined by ELISA, as a function of the spiking level is shown in Figure 5.8. The obtained linear regressions have correlation coefficients (*r*) of 0.990 and 1.000 and slopes of 0.81 for wastewaters after biological treatment collected at North and South WWTPs, respectively. Correlation coefficients and slopes close to 1 should be indicative that no significant

organic matter interferences are taking place as these interferences are usually non-linear and concentration dependent. Very good correlation coefficients were obtained, indicating a linear behavior. The slope values do not reflect an optimal case; however, they are consistent with average recoveries between 86 ± 13% and 97 ± 11%, which were considered satisfactory. Interestingly, the carbamazepine concentration in the samples before spiking, given by the y-intercept (0.61 ± 0.08 and 0.58 ± 0.01), is similar for the two different WWTPs. Note that, taking into account the complexity of the tested samples, these results should represent the worst case scenario of organic matter influences in the analyzed samples.

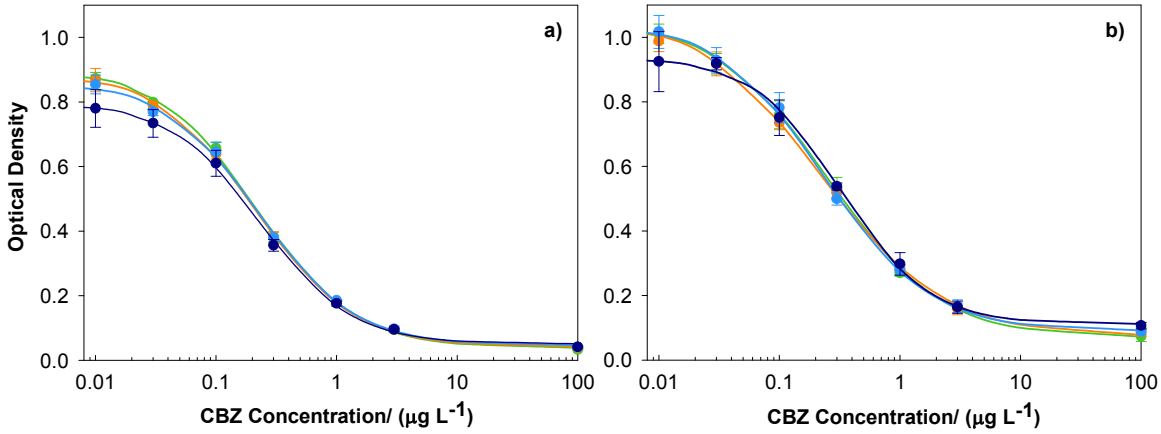

Figure 5.7. Evaluation of organic matter effects *a)* in the absence and *b)* in the presence of BSA, on the ELISA calibration curves, at pH 10.5. Carbamazepine calibrators in the absence of humic acids and in the presence of 0.1, 1.0 and 10.0 mg L^{-1} of humic acids, are represented in green, orange, blue and dark blue, respectively.

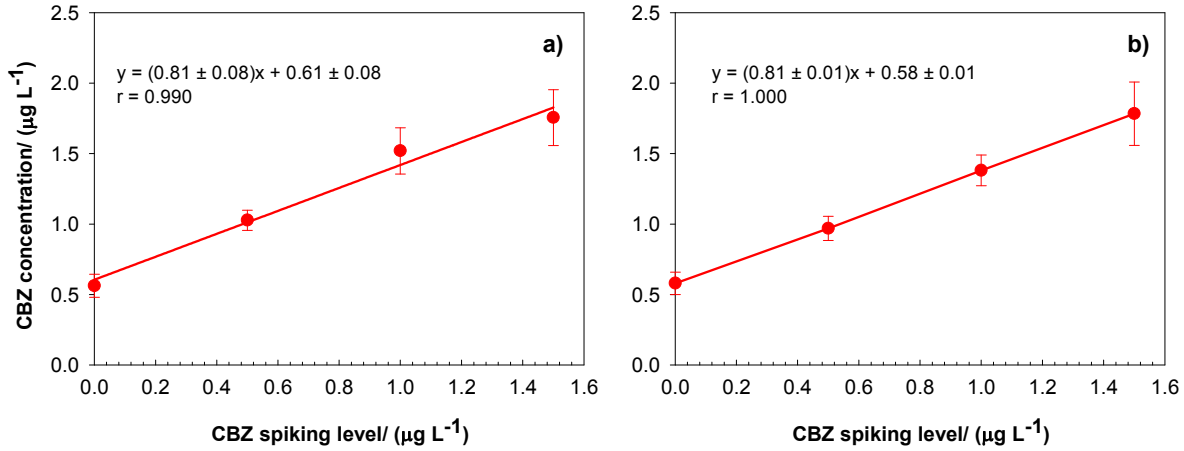

Figure 5.8. Carbamazepine concentrations determined by ELISA after spiking wastewater samples with 3 different concentrations of carbamazepine. *a)* and *b)* display results obtained with samples after biological treatment collected at North and South WWTPs, respectively.

5.3.3 Selection of the optimal ELISA conditions

The evaluation of different sample buffers in the assay´s performance allows choosing the optimal experimental conditions, considering the effects of dissolved organic matter, salinity and also cetirizine cross-reactivity. Table 5.2 presents a summary of the main aspects to take into account. Note that at pH 4.5, the antibody has more affinity for cetirizine than for carbamazepine and, for this reason, no matrix effects tests were performed under these conditions, as it was not considered to be the optimal pH value. In general, pH 7.6 yielded better results with lower turning points (thus, higher sensitivity), higher signal and also very good response to salinity and organic matter influences. However, at this pH, cross-reactivity with cetirizine is a major problem: the assay has roughly the same affinity towards both carbamazepine and cetirizine. In the light of the preliminary conclusions taken in section 5.3.1, where it was demonstrated that samples might be contaminated with cetirizine due to a systematic and significant difference on the results obtained at different pH, the sample buffer with pH 10.5 was selected as the optimal condition. At this pH, cross-reactivity with cetirizine is minimal and the results obtained for the matrix tests are still reasonable.

Table 5.2. Comparison of some characteristics of the carbamazepine ELISA at pH 7.6 and 10.5.

	pH 7.6 – TRIS buffer	pH 10.5 – Glycine buffer
Turning point	Low	High
Colorimetric signal	High	Low
Salinity effects	No significant effects up to 30 g L^{-1} of NaCl	Effects for [CBZ] < 0.1 μg L^{-1} might be significant
Organic matter effects	Satisfactory recoveries in the working range	Satisfactory recoveries in the working range
Cetirizine cross-reactivity	High	Low

5.3.4 Performance of the ELISA methodology at pH 10.5

An ELISA dose-response calibration curve and respective precision profile, at pH 10.5, are presented in Figure 5.9. In order to adequately define the quantitation/working range of the assay, 16 calibrators were assayed (6 replicates per calibrator). Considering a maximum allowable relative error of carbamazepine concentration of 30%, a quantitation range from 0.03 to 10 μg L^{-1} was determined. This quantitation range was defined not considering matrix effects. As stated before, salinity and organic matter might affect the test for carbamazepine concentrations below 0.1 μg L^{-1}.

This could imply a narrower quantitation range in case of samples with complex matrices, leading to the conclusion that the precision of the method not only depends on the carbamazepine concentration but also on the complexity of the sample.

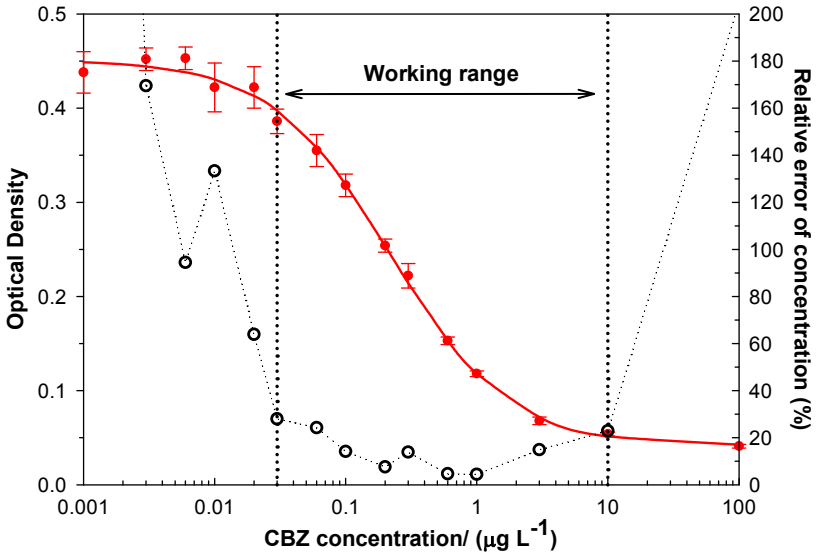

Figure 5.9. Calibration curve of ELISA (red circles) (r^2 = 0.999) and precision profile (open circles). The precision profile and determination of the relative error of concentration were calculated in accordance with Ekins (1981). A working range was defined between 0.03 and 10 µg L^{-1}, considering a maximum allowable relative error of concentration of 30%. All data obtained at pH 10.5.

5.3.5 Simultaneous determination of carbamazepine and cetirizine

As concluded before, the collected samples might also contain cetirizine. Depending on the pH value, the determination of carbamazepine by ELISA can be affected by a positive bias and hence, the relevance of this systematic error will depend on the concentration of the cross-reactant. At pH 10.5, with a molar cross-reactivity of 15%, this bias is not expected to be significant. However, it is interesting to consider that the analysis of samples at two different pH values allows taking into account the contribution of cetirizine to the total concentration of carbamazepine recognized by the assay. Therefore, this data can be considered as data from two immunoassays of different selectivity for a two-parameter set which enables the quantification of both carbamazepine and cetirizine in the samples. The approach here described is similar to the one applied by Schneider et al. (1992) for the quantification of two triazine herbicides using immunoassays of different selectivity. Quantification of carbamazepine and cetirizine in the samples was done by solving the following equations (equations 5.7 and 5.8):

$$C_{Total,\ pH4.5} = C_{CBZ} + CR_{pH4.5} \times C_{Cetirizine} \quad (5.7)$$

$$C_{Total,\ pH10.5} = C_{CBZ} + CR_{pH10.5} \times C_{Cetirizine} \quad (5.8)$$

where C_{CBZ} and $C_{Cetirizine}$ are the concentrations of carbamazepine and cetirizine in the samples, respectively; $CR_{pH4.5}$ and $CR_{pH10.5}$ are the cross-reactivities at pH 4.5 and 10.5, respectively and $C_{Total,\ pH4.5}$ and $C_{Total,\ pH10.5}$ are the total concentrations resulting from the assay, determined with the carbamazepine calibration curves, at pH 4.5 and pH 10.5, respectively.

In order to validate the proposed approach, contaminated surface water samples were spiked with 0.05 to 1.0 μg L^{-1} of carbamazepine, cetirizine or both and were analyzed at pH 4.5 and 10.5, by ELISA. Subsequently, equations 3 and 4 were applied to the raw experimental data to calculate carbamazepine and cetirizine concentrations. Results are presented in Table 5.3.

Table 5.3. ELISA response at pH 4.5 and 10.5 (± standard deviation, 3 replicates) and carbamazepine (CBZ) and cetirizine (CTRZ) extrapolated concentrations (using equations 3 and 4) for a surface water sample spiked with carbamazepine, cetirizine or both. Specific (mass-related) cross-reactivities of 298 and 14%, at pH 4.5 and 10.5, respectively, were used in the calculation. Mean recoveries, in percentage, are also shown.

Spiked concentration/ (μg L^{-1})		ELISA response/ (μg L^{-1}), n=3		Extrapolated concentrations/ (μg L^{-1})		Recoveries %	
CBZ	CTRZ	pH 4.5	pH 10.5	CBZ	CTRZ	CBZ	CTRZ
-	-	0.41 ± 0.05	0.29 ± 0.04	0.29	0.041	-	-
0.059	-	0.44 ± 0.07	0.34 ± 0.02	0.34	0.032	98	-
0.118	-	0.54 ± 0.02	0.41 ± 0.04	0.40	0.048	99	-
0.297	-	0.72 ± 0.03	0.63 ± 0.05	0.63	0.032	107	-
0.593	-	1.05 ± 0.03	0.91 ± 0.01	0.90	0.049	103	-
1.180	-	1.7 ± 0.1	1.46 ± 0.04	1.4	0.071	99	-
-	0.054	0.57 ± 0.06	0.30 ± 0.02	0.29	0.095	-	100
-	0.108	0.7 ± 0.1	0.32 ± 0.03	0.30	0.14	-	95
-	0.272	1.1 ± 0.2	0.35 ± 0.06	0.31	0.28	-	89
-	0.543	2.0 ± 0.2	0.38 ± 0.03	0.30	0.56	-	96
-	1.080	4.0 ± 0.4	0.43 ± 0.01	0.26	1.2	-	111
0.059	0.054	0.64 ± 0.06	0.36 ± 0.03	0.35	0.10	101	104
0.118	0.108	0.79 ± 0.02	0.43 ± 0.02	0.41	0.13	101	87
0.297	0.272	1.45 ± 0.02	0.65 ± 0.01	0.61	0.28	104	90
0.593	0.543	2.6 ± 0.2	0.97 ± 0.06	0.90	0.58	102	99
1.180	1.080	5.0 ± 0.6	1.6 ± 0.2	1.4	1.2	98	106

The calculated concentrations are consistent with average recoveries between 98 and 107% for carbamazepine and 87 and 111% for cetirizine. Also, a linear regression analysis of the analyte concentration in the spiked samples as a function of the spiking level enabled to conclude that a satisfactory linear behavior is obtained in all cases, with correlation coefficients ranging from 0.997 to 0.999 and slopes from 0.98 to 1.10. No significant differences were found in the linear response of the analyte concentration between samples spiked only with carbamazepine, only with cetirizine or both. These experiments allowed proving the reliability of the described approach to calculate the contribution of cetirizine in the ELISA response.

5.3.6 Quantification of carbamazepine in ground, surface and wastewaters by ELISA

The results of the analyses of environmental samples by ELISA, where carbamazepine was detected, are presented in Table 5.4 (raw data at pH 4.5 and 10.5 and extrapolated concentrations). The obtained results indicate that all the samples collected in WWTPs are contaminated with carbamazepine concentrations between 0.5 and 0.7 μg L^{-1} with relative standard deviations ranging from 11 to 19%. Carbamazepine concentrations did not decrease from stage to stage, indicating that the applied treatments (primary decantation, biological treatment and secondary decantation) are not efficient in the removal of this pharmaceutical. On the contrary, a possible increase of the carbamazepine concentration can be stated, especially in the South WWTP, which is fully compatible with previous studies indicating the possibility of deconjugation reactions in the sewage, causing an increase of the concentration of the deconjugated pharmaceutical (see chapter 2). Cetirizine was also found in all the samples from WWTPs with concentrations between 0.31 ± 0.04 and 0.60 ± 0.06 μg L^{-1} in the North WWTP and between 0.23 ± 0.04 and 0.38 ± 0.07 μg L^{-1} in the South WWTP. In opposition to carbamazepine, the concentration of cetirizine clearly decreases during the wastewater treatment process, specifically after undergoing biological treatment.

The presence of cetirizine shows that it may be necessary to perform the ELISA test at two pH values to be able to present more accurate carbamazepine concentrations. However, note that ELISA results at pH 10.5, presented in Tables 5.3 and 5.4, do not significantly differ from the carbamazepine concentrations obtained after accounting for the presence of cetirizine and, thus, in this case, the analysis at a single pH (10.5) gives accurate results. This is valid due to low cross-reactivity at pH 10.5 and as long as the concentration of cetirizine does not significantly exceed the concentration of carbamazepine. The information about the approximate ratio of these

pharmaceuticals in the samples becomes vital when large number of samples of unscreened areas need to be processed. It is important to highlight that performing the assay at two pH values doubles the work necessary but, on the other hand, also doubles the obtained data about the samples, as it enables the quantification of cetirizine.

Table 5.4. Carbamazepine (CBZ) and cetirizine (CTRZ) concentrations (± standard deviation) determined by ELISA (3 replicates) and LC-MS/MS (2 replicates) (only for carbamazepine). Raw data obtained at pH 4.5 and 10.5 are also shown. Samples were analyzed by both methodologies without any sample pretreatment. Omitted samples (7 ground and 10 surface waters) did not present detectable carbamazepine concentrations. Sampling site names are in accordance with designations presented in Figure 5.3.

Sampling site*	ELISA/ (μg L^{-1})				LC-MS/MS/ (μg L^{-1})
	Response at pH 4.5	Response at pH 10.5	[CBZ] (corrected)	[CTRZ] (corrected)	[CBZ]
SWW1	1.3 ± 0.1	0.5 ± 0.1	0.48 ± 0.07	0.38 ± 0.07	0.44 ± 0.01
SWW2	1.13 ± 0.09	0.6 ± 0.1	0.58 ± 0.08	0.24 ± 0.02	0.49 ± 0.01
SWW3	1.1 ± 0.2	0.6 ± 0.1	0.57 ± 0.06	0.23 ± 0.04	0.50 ± 0.01
NWW1	2.0 ± 0.2	0.7 ± 0.1	0.65 ± 0.08	0.60 ± 0.06	0.50 ± 0.01
NWW2	1.3 ± 0.1	0.6 ± 0.1	0.56 ± 0.08	0.31 ± 0.04	0.55 ± 0.02
NWW3	1.3 ± 0.1	0.6 ± 0.1	0.60 ± 0.07	0.31 ± 0.03	0.54 ± 0.01
S11	0.19 ± 0.05	0.11 ± 0.02	0.11 ± 0.02	0.04 ± 0.01	0.15 ± 0.01

*Sample description: SWW1 - South WWTP after primary treatment; SWW2 - South WWTP after biological treatment; SWW3 - South WWTP, final effluent; NWW1 - North WWTP after primary treatment; NWW2 - North WWTP after biological treatment; NWW3 - North WWTP, final effluent; S11 - Surface water of *Ria de Aveiro*, collected in a coastal touristic area.

Considering surface water samples, only 1 of the 11 sampling points is contaminated with 0.11 ± 0.02 μg L^{-1} of carbamazepine. The other surface waters as well as ground water samples did not have detectable levels of carbamazepine or cetirizine. Assuming that carbamazepine is, in fact, an adequate anthropogenic pollution marker, these results give a first indication that the coastal lagoon of *Ria de Aveiro* is not considerably polluted by domestic wastewaters.

5.3.7 Comparison between ELISA and LC-MS/MS

In order to validate results obtained by ELISA, samples were analyzed by LC-MS/MS. Samples were directly injected after filtration and addition of internal standard, as it was previously demonstrated that these procedures effectively compensate matrix effects (Bahlmann et al., 2009). Linear regression and correlation coefficient of the LC-MS/MS are presented in Table 5.5. LC-MS/MS results are presented in Table 5.4. Carbamazepine concentrations determined by ELISA are, in general, 2 to 28% higher than the LC-MS/MS results. This overestimation is presumably caused by the mix contribution of minor matrix effects and the presence of other cross-reactants such as epoxy-carbamazepine or 2-hydroxy-carbamazepine. Some relevant metabolites of carbamazepine were also simultaneously determined by LC-MS/MS and results are shown in Table 5.6. Di-OH-carbamazepine is the metabolite with the highest concentration but it is also the metabolite with the lowest cross-reactivity (Table 5.1). The metabolites with the highest cross-reactivity are epoxy-carbamazepine and 2-OH-carbamazepine and were positively identified in the samples with concentrations around 0.05 and 0.1 μg L^{-1}, respectively.

The determination of the cetirizine concentration in the samples appeared as a consequence of the ELISA methodology and the results were not validated by LC-MS/MS. Although not quantified, the presence of cetirizine in the samples was positively identified by the LC-MS/MS chromatograms.

Table 5.5. Linear regression equation and correlation coefficients (*r*) of the LC-MS/MS methodology for carbamazepine (CBZ) and some metabolites.

	Linear regression equation	*r*
CBZ	y = (7.31 ± 0.01) x + 0.045 ± 0.005	1.000
Epoxy-CBZ	y = (1.540 ± 0.006) x - 0.003 ± 0.002	0.9999
2-OH-CBZ	y = (3.14 ± 0.02) x - 0.007 ± 0.006	0.9999
3-OH-CBZ	y = (4.44 ± 0.03) x - 0.01 ± 0.01	0.9999
10-OH-CBZ	y = (2.18 ± 0.02) x - 0.007 ± 0.007	0.9998
DiOH-CBZ	y = (0.270 ± 0.003) x - 0.001 ± 0.001	0.9997

Table 5.6. Concentration (μg L^{-1} ± standard deviation) of the main carbamazepine metabolites determined by LC-MS/MS (2 replicates). Sampling site names are in accordance with designations presented in Figure 5.3.

Sampling site	Epoxy-CBZ	2-OH-CBZ	3-OH-CBZ	10-OH-CBZ	DiOH-CBZ
SWW1	0.054 ± 0.005	0.081 ± 0.004	0.089 ± 0.001	0.191 ± 0.002	1.6 ± 0.1
SWW2	0.05 ± 0.01	0.10 ± 0.01	0.089 ± 0.007	0.211 ± 0.003	1.78 ± 0.05
SWW3	0.056 ± 0.001	0.10 ± 0.01	0.08 ± 0.01	0.226 ± 0.004	1.74 ± 0.05
NWW1	-	0.10 ± 0.01	0.09 ± 0.02	0.34 ± 0.02	2.1 ± 0.2
NWW2	0.042 ± 0.007	0.106 ± 0.009	0.106 ± 0.003	0.301 ± 0.007	2.2 ± 0.2
NWW3	0.044 ± 0.002	0.096 ± 0.005	0.11 ± 0.01	0.31 ± 0.02	2.27 ± 0.01
S11	-	-	-	-	-

In general, results obtained by ELISA were considered highly satisfactory. As stated before, running the ELISA test at two different pH values doubles the effort and decreases the time efficiency of this methodology. However, even in this worst-case scenario, the proposed methodology presents some interesting advantages when compared to LC-MS/MS. This methodology is much cheaper concerning costs involved in equipment and maintenance. Moreover, it is still feasible to analyze dozens of samples in a few hours, allowing to perform large environmental screenings. ELISA and LC-MS/MS should be considered as two complementary techniques, with different applicability and purposes. Combining both methods, when large amounts of samples need to be processed, enables, in a first step, to carry out large screenings to identify contaminated areas by ELISA followed by reanalysis of specific samples or questionable results by LC-MS/MS. It is not reasonable to consider LC-MS/MS as an adequate approach to large preliminary screenings as well as ELISA might not be the most adequate technique to use in situations that demand extremely accurate analysis.

5.4 CONCLUSIONS

In this study, a previously developed immunochemical method was applied to the quantification of carbamazepine in aqueous environmental samples.

The assay has proven to be adequate for the analysis of complex matrix samples, namely with high concentrations of dissolved organic matter and sodium chloride. The implementation of this methodology revealed that samples as complex as wastewaters (even at the initial stage of the treatment process) can be analyzed without any further sample cleanup, except from a filtration step. The optimal conditions of the assay were chosen taking into consideration the pH dependent cross-reactivity to the pharmaceutical cetirizine, which was found to be present in surface and wastewaters contaminated with carbamazepine, in the Portuguese region studied. To overcome this problem, it is possible to perform the assay at pH 10.5 (conditions at which the cross-reactivity is minimal) or to perform the assay at two pH values allowing to correct the carbamazepine concentrations and to determine cetirizine in the samples, too.

Validation with LC-MS/MS showed that ELISA results were 2 to 28% overestimated. The obtained results highlight that the developed methodology is adequate to perform large environmental screenings in samples with distinct matrices, being a valid alternative and/or complementary methodology to the reference chromatographic techniques, in the context of high-throughput analysis.

Relative to the carbamazepine concentrations found, the results are in accordance with available literature data from other countries: carbamazepine is not effectively removed during wastewater treatments. In the particular case of the studied WWTPs, the final effluent showed carbamazepine concentrations around 0.6 μg L^{-1}. Also, it is possible to conclude that no significant differences were found in the concentration of carbamazepine of samples collected at different phases of the treatment, reinforcing the established inadequacy and quasi-total inefficiency of these removal stages with respect to this pharmaceutical. From 6 ground water and 11 surface water samples, only 1 surface water was contaminated with carbamazepine which demonstrates that the lagoon of *Ria de Aveiro* is not facing serious issues of contamination with domestic wastewaters.

5.5 REFERENCES

Bahlmann, A., Falkenhagen, J., Weller, M.G., Panne, U., Schneider, R.J., **2011**. Cetirizine as pH-dependent cross-reactant in a carbamazepine-specific immunoassay. *Analyst* 136, 1357-1364.

Bahlmann, A., Weller, M.G., Panne, U., Schneider, R.J., **2009**. Monitoring carbamazepine in surface and wastewaters by an immunoassay based on a monoclonal antibody. *Anal. Bioanal. Chem.* 395, 1809-1820.

Buchberger, W.W., **2007**. Novel analytical procedures for screening of drug residues in water, waste water, sediment and sludge. *Anal. Chim. Acta* 593, 129-139.

Carvalho, J.J., Weller, M., Panne, U., Schneider, R. J., **2010**. A highly sensitive caffeine immunoassay based on a monoclonal antibody. *Anal. Bioanal. Chem.* 396, 2617-2628.

Ekins, R., **1981**. The "precision profile": its use in RIA assessment and design. *The Ligand Quarterly* 4, 33-44.

Findlay, J.W.A., Dillard, R.F., **2007**. Appropriate calibration curve fitting in ligand binding assays. *Aaps J.* 9, E260-E267.

Gault, V.A., McClenaghan, N.H., **2009**. *Understanding Bioanalytical Chemistry - Principles and Applications*, 1st ed. Wiley-Blackwell.

Harlow, E., Lane, D., **1999**. *Using Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press.

Huerta-Fontela, M., Galceran, M.T., Ventura, F., **2010**. Fast liquid chromatography-quadrupole-linear ion trap mass spectrometry for the analysis of pharmaceuticals and hormones in water resources. *J. Chromatogr. A* 1217, 4212-4222.

Huo, S.M., Yang, H., Deng, A.P., **2007**. Development and validation of a highly sensitive ELISA for the determination of pharmaceutical indomethacin in water samples. *Talanta* 73, 380-386.

Infarmed, **2008**. Estatística do Medicamento - Autoridade Nacional do Medicamento e Produtos de Saúde (Portugal); http://www.infarmed.pt/portal/page/portal/INFARMED/PUBLICACOES/TEMATICOS/ESTATISTICA_MEDICAMENTO.

Infarmed, **2009**. Estatística do Medicamento - Autoridade Nacional do Medicamento e Produtos de Saúde (Portugal); http://www.infarmed.pt/portal/page/portal/INFARMED/PUBLICACOES/TEMATICOS/ESTATISTICA_MEDICAMENTO.

Jonkers, N., Sousa, A., Galante-Oliveira, S., Barroso, C.M., Kohler, H.P.E., Giger, W., **2010**. Occurrence and sources of selected phenolic endocrine disruptors in Ria de Aveiro, Portugal. *Environ. Sci. Pollut. R.* 17, 834-843.

Kalman, J. and Turner, A., **2007**. An evaluation of metal bioaccessibility in estuarine sediments using the commercially available protein, bovine serum albumin. *Mar. Chem.* 107, 486-497.

Kosonen, J., Kronberg, L., **2009**. The occurrence of antihistamines in sewage waters and in recipient rivers. *Environ. Sci. Pollut. R.* 16, 555-564.

Law, B. (Ed.), **2005**. *Immunoassay - A Practical Guide*. Taylor and Francis, London.

Leclercq, M., Mathieu, O., Gomez, E., Casellas, C., Fenet, H. and Hillaire-Buys, D., **2009**. Presence and fate of carbamazepine, oxcarbazepine, and seven of their metabolites at wastewater treatment plants. *Arch. Environ. Con. Tox.* 56, 408-415.

Loos, R., Locoro, G., Comero, S., Contini, S., Schwesig, D., Werres, F., Balsaa, P., Gans, O., Weiss, S., Blaha, L., Bolchi, M., Gawlik, B.M., **2010**. Pan-European survey on the occurrence of selected polar organic persistent pollutants in ground water. *Water Res.* 44, 4115-4126.

Madureira, T.V., Barreiro, J.C., Rocha, M.J., Cass, Q.B., Tiritan, M.E., **2009**. Pharmaceutical trace analysis in aqueous environmental matrices by liquid chromatography-ion trap tandem mass spectrometry. *J. Chromatogr. A* 1216, 7033-7042.

Madureira, T.V., Barreiro, J.C., Rocha, M.J., Rocha, E., Cass, Q.B., Tiritan, M.E., **2010**. Spatiotemporal distribution of pharmaceuticals in the Douro River estuary (Portugal). *Sci. Total Environ*. 408, 5513-5520.

Miao, X.-S. and Metcalfe, C.D., **2003**. Determination of carbamazepine and its metabolites in aqueous samples using liquid chromatography-electrospray tandem mass spectrometry. *Anal. Chem*. 75, 3731-3738.

Miao, X.-S., Yang, J.-J. and Metcalfe, C.D., **2005**. Carbamazepine and its metabolites in wastewater and in biosolids in a municipal wastewater treatment plant. *Environ. Sci. Technol.* 39, 7469-7475.

Nunes, G.S., **2005**. Immunochemical methods for analysis of environmental contaminants: Concepts, state of the art and perpectives. *Quim. Nova* 28, 462-471.

O'Malley, A.J., **2008**. A Bayesian precision profile for measuring the quality of immunoassay experiments. *Mathematical Physical and Engineering Sciences* 366, 2301-2312.

Ribeiro, C., Pardal, M.A., Martinho, F., Margalho, R., Tiritan, M.E., Rocha, E., Rocha, M.J., **2009a**. Distribution of endocrine disruptors in the Mondego River estuary, Portugal. *Environ. Monit. Assess*. 149, 183-193.

Ribeiro, C., Pardal, M.A., Tiritan, M.E., Rocha, E., Margalho, R.M., Rocha, M.J., **2009b**. Spatial distribution and quantification of endocrine-disrupting chemicals in Sado River estuary, Portugal. *Environ. Monit. Assess.* 159, 415-427.

Ribeiro, C., Tiritan, M.E., Rocha, E., Rocha, M.J., **2009c**. Seasonal and spatial distribution of several endocrine-disrupting compounds in the Douro river estuary, Portugal. *Arch. Environ. Con. Tox.* 56, 1-11.

Schneider, R.J., **2003**. Environmental immunoassays. *Anal. Bioanal. Chem.* 375, 44-46.

Schneider, R.J., Weil, L., Niessner, R., **1992**. Identification of two triazine herbicides in top soil layers using immunoassays of different selectivity. *Fresen. J. Anal. Chem.* 343, 145-146.

Shelver, W.L., Shappell, N.W., Franek, M., Rubio, F.R., **2008**. ELISA for sulfonamides and its application for screening in water contamination. *J. Agric. Food Chem.* 56, 6609-6615.

SimRia, www.simria.pt. (Accessed March **2011**)

Sousa, A., Schonenberger, R., Jonkers, N., Suter, M.J.F., Tanabe, S., Barroso, C.M., **2010**. Chemical and biological characterization of estrogenicity in effluents from WWTPs in Ria de Aveiro (NW Portugal). *Arch. Environ. Con. Tox.* 58, 1-8.

van Emon, J.M. (Ed.), **2007**. *Immunoassay and Other Bioanalytical Techniques*. CRC Press, Taylor and Francis, Boca Raton.

van Emon, J.M., Chuang, J.C., Lordo, R.A., Schrock, M.E., Nichkova, M., Gee, S.J., Hammock, B.D., **2008**. An enzyme-linked immunosorbent assay for the determination of dioxins in contaminated sediment and soil samples. *Chemosphere* 72, 95-103.

CHAPTER 6

Adsorption of carbamazepine onto agricultural soils

In this chapter, the sorption behavior of carbamazepine onto agricultural soils was studied. For this purpose, batch equilibrium experiments were performed using soils subjected to distinct long-term fertilizations. The adsorption experiments were followed by UV spectral deconvolution and the results compared with those from micellar electrokinetic chromatography. It was possible to conclude that results obtained by both methods did not present significant statistical differences at 95% confidence level, proving that, in this context, UV spectral deconvolution is a simple and fast procedure adequate to follow sorption experiments. The adsorption of carbamazepine onto the selected soils was satisfactorily described by the Freundlich equation; the Freundlich adsorption coefficients (K_F) (between 2.1 ± 0.2 and 5.0 ± 0.3 $mg^{1-N}\ L^N\ Kg^{-1}$) indicate that the adsorption behavior of carbamazepine is dependent on the soil fertilization. Also, this anti-epileptic pharmaceutical is not extensively adsorbed and thus, a significant percentage of carbamazepine appears to remain in the aqueous phase. Moreover, it is expectable that contaminated soils constitute a potential source of contamination for surface and ground waters through run-off and infiltration.

CONTENTS

6.1 CONTEXTUALIZATION

The study of the sorption behavior of pharmaceuticals is of great relevance to understand the fate of these compounds in the water/soil (or sediment) interface and constitute an indispensable element to evaluate their real environmental impact.

In this chapter, the adsorption of carbamazepine onto agricultural soils subjected to long-term fertilization experiments was studied. This research aims to evaluate the effects of distinct soil amendments (and, thus, different soil organic matter contents and characteristics) on the adsorption of carbamazepine and to assess if carbamazepine contaminated soils constitute a potential source of contamination of adjacent waters. Additionally, the results presented in this chapter also aim at assessing the adequacy of a simple and fast UV spectral deconvolution (UVSD) method to follow sorption experiments. The validation of the proposed methodology was done by micellar electrokinetic chromatography (MEKC), used as reference technique. A brief introduction to the main concepts involved in this study is presented below.

6.1.1 The environmental relevance of sorption studies

The literature review previously presented highlighted that WWTPs are the main vehicle for the introduction of pharmaceuticals in the environment through the discharge of contaminated effluents. However, and apart from discharge in surface waters, WWTPs effluents are frequently used for irrigation of agricultural fields, especially in arid or semiarid areas where they are considered an important water resource (Pedersen et al., 2005; Williams et al., 2006; Glassmeyer et al., 2008; Gielen et al., 2009). Additionally, the sludge produced in WWTPs is also commonly applied in agricultural fields as a soil amendment, taking advantage of its organic matter rich matrix to improve soil properties (Carmosini and Lee, 2008; Wu et al., 2010). This reuse of the effluents and sludge reduces the demand for water and fertilizer resources which is particularly relevant in the case of irrigation waters that account for roughly 65% of the total water needs worldwide (Levine and Asano, 2004). Despite the referred benefits, these practices constitute the most important pathways for the introduction of pharmaceuticals into soils which might then contribute to the contamination of surface and ground waters through run-off, leaching and overland flow (Pedersen et al., 2005; Wu et al., 2010).

The study of the sorption behavior of pharmaceuticals that are frequently found in WWTPs effluents and sludge is essential for the understanding of their mobility between different

environmental compartments and, consequently, the main degradation processes that will affect their persistence in such compartments. In the specific case of carbamazepine, a potential anthropogenic pollution indicator, sorption studies are even more relevant. Pharmaceuticals with high adsorption affinity cannot be considered adequate anthropogenic pollution indicators due to their high preference to adsorb onto the solid matrix. Therefore, low to medium adsorption affinities are a pre-requisite for a pharmaceutical to be considered a marker of anthropogenic pollution in aqueous environments.

6.1.2 Sorption experiments and isotherms

Sorption studies are usually performed using batch or column displacement methods (Limousin et al., 2007; Carmosini and Lee, 2008). Column displacement methods involve the evaluation, over time, of the movement of a pollutant through a soil column and the outflow concentration of the pollutant *versus* time is analyzed (Carmosini and Lee, 2008). These methods were not used in the sorption experiments hereby described and are, thus, out of the scope of this contextualization. On the other hand, batch experiments, applied in this study, consist on shaking the soil/sediment with a solution containing the pollutant of interest until the adsorption/desorption equilibrium is reached. The equilibrium concentration of the pollutant in the aqueous/solid phases is then measured (Limousin et al., 2007). Using this approach, multiple concentrations of the pollutant are tested using a defined amount of solid phase. The soil/sediment mass to solution volume ratio should be chosen so that 40-60% of sorption is attained at the end of the experiment. The sorption data are then analyzed by plotting the concentration of pollutant in the solid phase against the concentration which remained in solution, and a sorption isotherm, which characterizes the sorption affinity of the analyte, is obtained (Carmosini and Lee, 2008).

In the literature, sorption isotherms are most commonly described by one of the following models (Carmosini and Lee, 2008):

a) Linear model:

$$Q_e = K_d \times C_e \quad (6.1)$$

where K_d is the linear distribution coefficient (L Kg^{-1}), Q_e and C_e are the analyte concentration in the solid phase (mg Kg^{-1} or mol Kg^{-1}) and in the aqueous phase (mg L^{-1} or mol L^{-1}), respectively.

b) Freundlich model:

$$Q_e = K_F \times C_e^N \quad (6.2)$$

where K_F is the Freundlich sorption coefficient ($mg^{1-N}\ L^{N}\ Kg^{-1}$ or $mol^{1-N}\ L^{N}\ Kg^{-1}$) and N defines the isotherm non-linearity (dimensionless). Note that when N equals 1, this equation is equivalent to the linear model of equation 6.1.

c) Langmuir model:

$$Q_e = \frac{Q_{e,max} \times K_L \times C_e}{1 + K_L \times C_e} \quad (6.3)$$

where K_L is the Langmuir affinity coefficient (L mg^{-1} or L mol^{-1}) and $Q_{e,max}$ defines the maximum adsorption capacity (mg Kg^{-1} or mol Kg^{-1}).

The linear model describes a sorption behavior in which the ratio between the concentration of the analyte in the aqueous and solid phases is constant at any concentration level. Conversely, the non-linear models describe isotherms characterized by a decrease of the ratio between solid and aqueous phase concentrations with the increase of the analyte concentration. The presented non-linear models fit two distinct situations: (a) the curve reaches a plateau, indicating that the soil/sediment achieved saturation, and a maximum adsorption capacity is defined – Langmuir equation and (b) a plateau is never achieved and, accordingly, the solid phase does not clearly exhibits a limited sorption capacity (Aboul-Kassim and Simoneit, 2001; Limousin et al., 2007). On the whole, for all these models, the relation between Q_e and C_e established by the isotherm implies that the adsorption/desorption processes have reached an equilibrium and that all the physico-chemical parameters of the system are constant (Limousin et al., 2007).

The comparison between sorption coefficients obtained by different studies or with different soils must be carefully interpreted. For instance, when comparing sorption coefficients determined for different soils and the isotherms have different linearity, a concentration specific distribution coefficient (K_d^*) - at a chosen concentration within the experimental range tested - should be determined from the non-linear coefficients. K_d^* is then independent of the linearity of the isotherm; this parameter can be determined using the following equation (Carmosini and Lee, 2008):

$$K_d^* = K_F \times C_e^{N-1} \quad (6.4)$$

Moreover, when comparing results from different soils/sediments, their organic matter content should be taken into account, as it influences the sorption process, especially in the case of highly hydrophobic analytes. Sorption coefficients independent of the organic carbon content can be obtained by determining the ratio between the sorption coefficient and the percentage of the total organic carbon of the solid phase (Aboul-Kassim and Simoneit, 2001).

6.1.3 UV spectral deconvolution

The use of techniques such as HPLC and capillary electrophoresis to follow sorption studies implies, in most cases, performing complex sample preparation protocols and/or time consuming analysis. The high number of samples that result from batch experiments demands the application of less expensive and more time efficient procedures. In this context, an UVSD method was proposed as a simple and fast alternative to follow sorption experiments.

UVSD was applied for the first time to environmental related analysis in 1993 by Thomas et al. (1993) and since then several other studies have been published (Escalas et al., 2003; Coulomb et al., 2006; Hassouna et al., 2007; Kibbey et al., 2009; Lima et al., 2010). The principle of UVSD is based on the assumption that the total absorbance spectrum of a mixture can be decomposed in a few reference spectra; on other words, the spectrum of a mixture can be considered as a linear combination of the spectra of its individual components (Thomas et al., 1993). Hence, this approach allows the quantification of the components of a mixture without the need to perform their physical separation. However, this is only feasible if all the components of the mixture are known and if it is possible to obtain a UV spectrum of each of them individually.

Considering the UV spectra of the individual components of a mixture (referred to as references), the quantification of a given analyte in the solution is then possible by determining its coefficient in the linear combination, by means of the following equation:

$$S_{OD} = \sum_{i=1}^{p} a_i \times REF_i(\lambda) \pm r \quad (6.5)$$

where S_{OD} is the sample optical density, p is the number of the reference spectra, a_i is the coefficient of the i^{th} reference spectrum in the linear combination, $REF_i(\lambda)$ is the absorbance of the i^{th} reference spectrum at the wavelength λ and r is the error at each wavelength (Hassouna et al., 2007; Nam et al., 2008; Lima et al., 2010).

The implementation of this principle in sorption experiments allows a huge increase of the time efficiency of the whole procedure. In this study, the validity of the UVSD results will be assessed by MEKC analysis.

6.2 MATERIALS AND METHODS

6.2.1 Chemicals and Instrumentation

All chemicals used were of analytical grade: carbamazepine (99%, Sigma), sodium dodecylsulphate (99%, for electrophoresis, Sigma-Aldrich), hexadimethrine bromide (polybrene, Sigma-Aldrich), sodium chloride, ethylvanillin (99%, Aldrich), sodium tetraborate (Riedel-de Haën), acetonitrile (HPLC gradient grade, VWR, Prolabo) and methanol (HPLC grade). All the solutions (buffers and standard solutions) were prepared in ultra-pure water, obtained using a Milli-Q Millipore system (Milli-Q plus 185).

The capillary electrophoresis analyses were performed using a commercial instrument (Beckman P/ACE MDQ (Fullerton, CA, USA)), equipped with a UV-Vis photodiode array detection system. UV spectra were obtained using a UV-Vis Shimadzu spectrophotometer. Adsorption experiment samples were shaken using an overhead shaker (Heidolph, Reax 2) at 50 rpm. Samples were centrifuged using a Selecta centrifuge (Mixtasel, Model 540) at 4 000 rpm.

6.2.2 Soil Samples

The soil samples (0 – 30 cm) were collected from agricultural fields subjected to a long-term experiment (since 1962) at the experimental farm of INRES – Institute of Plant Nutrition, University of Bonn, Germany. The soils studied in this investigation were subjected to different fertilizations: MIN (mineral fertilization), SLU (sewage sludge from a WWTP) and COM (compost from organic household waste) and were characterized in a previous study (Lima et al., 2009). Total organic matter (TOM) contents were determined by loss on ignition of portions of the soil at 550 ºC for 4 h, and total organic carbon (TOC) contents were determined by means of dry combustion - results shown in Table 6.1 (Lima et al., 2009). Prior to sorption studies, the soils were air dried and passed through a sieve with mesh size of 2 mm.

Table 6.1. Total organic matter (TOM) and total organic carbon (TOC), in percentage, of the studied soils, as presented in Lima et al. (2009).

	TOM %, (n=3)	TOC %
MIN	5.3 ± 0.3	1.24
SLU	6.2 ± 0.3	1.98
COM	9.4 ± 1.0	2.76

6.2.3 MEKC analysis

The MEKC methodology, used as reference to validate UVSD results, was adapted from the work presented in chapter 3.

6.2.3.1 Capillary column conditioning and coating

Briefly, a fused-silica capillary with a total length of 50 cm (40 cm to detector) and 75 μm of internal diameter was used. Approximately 1 mm of the capillary external coating was removed by burning the extremities prior to conditioning; the capillary extremities were then polished to decrease the baseline noise. New capillaries were conditioned with 1 M NaOH for 30 min followed by ultra-pure water for 15 min. Subsequently, the capillary was coated with hexadimethrine bromide (polybrene) 0.5% (*w/v*) in 0.5 M NaCl, by flushing it for 20 min. Finally, the capillary was washed with ultra-pure water for 2 min followed by running buffer for 20 min. The running buffer consisted on 25 mM borax and 50 mM SDS, pH 9.2, freshly prepared every 2 days and stored at 4 ºC.

6.2.3.2 Separation conditions

Samples and standard solutions were injected for 4 s at 0.5 psi. Electrophoretic separations were performed in direct polarity with a positive potential supply of 20 kV for 13 min. The temperature of the capillary was maintained at 25 ºC. Detection of carbamazepine was monitored at 210 nm. Running buffer vials were changed every 6 runs.

6.2.3.3 Analysis of standard solutions and samples

A stock solution of ethylvanillin was used as internal standard (IS). This solution was prepared by dissolving ethylvanillin in acetonitrile (approximately 10% of the final volume) and further diluting it with ultra-pure water to a final concentration of 167 mg L^{-1}. IS was added to standard solutions and samples to a final concentration of 3.34 mg L^{-1}. The MEKC calibration curve was obtained by analyzing seven standard solutions of carbamazepine in quadruplicate, with concentrations ranging from 0.50 to 10.00 mg L^{-1}.

6.2.4 UVSD analysis

UVSD was performed using Microsoft Excel®. The output data consisted on the coefficient of each component in the linear combination (carbamazepine, organic matter and background), statistical parameters involving the determination of the coefficients and deconvolution residuals.

Firstly, the UV spectra of the reference solutions (containing each sample component separately) were obtained. Reference solutions consisted on (1) $CaCl_2$ 0.005 M, (2) a carbamazepine standard solution 2.00 mg L^{-1} in $CaCl_2$ and (3) raw extract of the soil organic matter. The organic matter raw extract was obtained by shaking tubes containing the soil under study and a $CaCl_2$ solution 0.01 M (ratio 1:2 (*w/v*)) during 4 h at 24 ± 1 ºC. The tubes were centrifuged and the supernatant filtered and diluted by a factor of 2 to avoid matrix effects on the UVSD analysis due to high concentration of organic matter.

UVSD calibration curves were obtained by analyzing 7 standard solutions with concentrations ranging from 0.10 to 5.00 mg L^{-1}. The standard solutions were constituted by the analyte and raw organic matter extract in 0.01 M $CaCl_2$ diluted by a factor of two. Samples were centrifuged, filtered and diluted two times prior to analysis.

UV spectra of reference solutions, standard solutions and samples were performed using microcells from Brand, with 1 cm of light path. Spectra were registered between 230 and 320 nm for MIN and SLU soils and from 235 to 320 nm for COM soil. A slightly narrower wavelength range was selected for COM soil in order to avoid matrix effects due to the very high concentration of organic matter. All spectra were obtained in triplicate.

The UVSD method described above was adapted from Lima et al. (2010).

6.2.5 Adsorption experiments

The adsorption studies were performed according to the recommendations described in the OECD guideline nº 106 (OECD, 2000).

A stock solution of carbamazepine (10.0 g L^{-1}) was prepared in methanol and a solution with an intermediate concentration (0.10 g L^{-1}) was prepared by diluting the stock solution in ultra-pure water. The carbamazepine solutions used in the adsorption experiments were prepared by diluting the 0.10 g L^{-1} carbamazepine solution in $CaCl_2$ 0.01 M.

6.2.5.1 Preliminary studies and suitability of MEKC and UVSD methods

Prior to the determination of carbamazepine adsorption isotherm, some preliminary experiments were undertaken in order to determine the time needed to attain the adsorption equilibrium. For this purpose, polypropylene tubes with a soil solution ratio of 1:2 (*w/v*) were shaken for different periods of time (from 15 min to 48 h) and analyzed by MEKC.

Some experiments to test the suitability of the adopted methodologies were also performed. To test the stability of carbamazepine and the possibility of adsorption to the polypropylene tubes, carbamazepine solutions in 0.01 M $CaCl_2$ were shaken during 4 h at the same conditions of the adsorption experiments. The samples were centrifuged, filtered and analyzed as described above. Also, tubes with a soil solution ratio of 1:2 (*w/v*) were shaken for 4 h with a 0.01 M $CaCl_2$ solution (without the test substance). Afterwards, and in order to investigate possible matrix effects, the samples were centrifuged, filtered and spiked with carbamazepine to a final concentration of 5 mg L^{-1} and subsequently analyzed. For this series of tests, recovery rates were obtained for both MEKC and UVSD analysis. All the described experiments were made in triplicate and samples were analyzed within the next two days.

6.2.5.2 Batch experiments

Adsorption studies of carbamazepine onto the three selected soils were made using batch equilibrium experiments. Five carbamazepine solutions with concentrations ranging from 2.00 to 10.0 mg L^{-1} were prepared in 0.01 M $CaCl_2$. The solutions with concentrations ranging from 2.00 to 8.00 mg L^{-1} were spiked with methanol so as to level out the methanol percentages in all the samples, as it was previously established that it influences the adsorption of the analyte onto soils. Accordingly, the final concentration of methanol in all samples was set to 0.1%. For each concentration, the carbamazepine solution was added to polypropylene tubes containing a soil sample so as to attain a final soil to solution ratio of 1:2 (*w/v*). Tubes containing carbamazepine solutions in the absence of the soil were included for control purposes. The tubes were shaken (head over head) at 50 rpm for 4 hours at 24 ± 1º C, centrifuged at 4 000 rpm for 15 min and filtered through a 0.22 μm membrane filter. Subsequently, samples were analyzed, in triplicate, by MEKC and UVSD.

6.3 RESULTS AND DISCUSSION

6.3.1 MEKC and UVSD calibration curves

For MEKC, a linear calibration curve was obtained using 7 standard solutions with concentrations ranging from 0.50 to 10.0 mg L^{-1}, by means of a least-squares linear regression based on the mean ratio between the peak area of carbamazepine and the peak area of the IS as a function of the standard solution concentration. Each standard solution was analyzed in quadruplicate; relative standard deviations of areas from repeated injections were below 2%.

The UVSD calibration curve was obtained by analyzing 7 standard solutions with concentrations ranging from 0.10 to 5.00 mg L^{-1}. The standard solutions were prepared in the same matrix of the samples obtained by batch experiments. The results were fitted to a least-squares linear regression by plotting the coefficient of carbamazepine in the linear combination of the spectra of the individual components as a function of the carbamazepine concentration. The calibration procedure was performed for each soil, as the composition of the standard solution depends on the soil. Each spectrum was obtained in triplicate and relative standard deviations of the carbamazepine coefficient in the linear combination were below 2.5%.

The statistical parameters of the calibration curves obtained by MEKC and UVSD are shown in Table 6.2. Both methodologies have very good correlation coefficients, confirming the good linear response of the methods in the studied range of concentrations. Limit of detection (LOD) and limit of quantification (LOQ) were also determined by taking $3s_{x/y}/b$ and $10s_{x/y}/b$, respectively, where b is the slope and $s_{x/y}$ is the residual standard deviation of the determined linear regression (J.N. Miller and Miller, 2005). Similar results were obtained for both methods, with the exception of the LOQ value for the MIN soil, determined by UVSD, which is slightly higher than the value obtained by MEKC or the LOQ values obtained for other soils. Nevertheless, the adopted methods presented satisfactory statistical figures.

Table 6.2. Linear regression equations, correlation coefficients (*r*), limits of detection (LOD) and limits of quantification (LOQ) of the MEKC and UVSD methods, considering the indicated concentration range.

Linear Regression	*r*	LOD/ (mg L^{-1})	LOQ/ (mg L^{-1})	Conc. range/ (mg L^{-1})
MEKC				
y = (0.813 ± 0.002) x – (0.026 ± 0.008)	1.0000	0.050	0.104	0.50 – 10.0
UVSD - SLU				
y = (0.488 ± 0.004) x + (0.00 ± 0.01)	0.9998	0.05	0.17	0.10 – 5.00
UVSD - COM				
y = (0.465 ± 0.003) x + (0.019 ± 0.009)	0.9998	0.064	0.189	0.10 – 5.00
UVSD - MIN				
y = (0.483 ± 0.007) x + (0.01 ± 0.02)	0.9994	0.09	0.31	0.10 – 5.00

6.3.2 Preliminary studies and suitability of MEKC and UVSD to follow the adsorption experiments

Preliminary experiments had shown that a soil solution ratio of 1:2 (*w/v*) was adequate to achieve between 40 and 60% of adsorption of the initial liquid-phase concentration; hence, this ratio was adopted to perform the subsequent experiments.

The experiments concerning the adsorption kinetics of carbamazepine enabled to conclude that the adsorption equilibrium was quickly reached, attaining approximately 55% of adsorbed analyte. No significant differences were found between the amount of carbamazepine adsorbed after 15 min or after 48 h of shaking. Based on these results, a reasonable 4 h period was established for performing batch experiments.

The suitability of MEKC and UVSD to analyze batch experiment samples was also tested. This procedure was intended to evaluate the existence of matrix effects capable of significantly influencing the performance of the methods. The raw organic matter extract (in 0.01 M $CaCl_2$) of the SLU soil was spiked with carbamazepine (at concentration levels of 5.0 mg L^{-1} and 2.5 mg L^{-1} for MEKC and UVSD, respectively). The solutions were subsequently analyzed and mean recoveries (n=3) of 95 ± 3% and 90.2 ± 0.7% were obtained for MEKC and UVSD, respectively. Additionally, and in order to test the stability of carbamazepine during batch experiments or even the adsorption of carbamazepine onto the polypropylene tubes, control samples (in the absence of the soils) were analyzed. Table 6.3 presents the average recoveries obtained by both methodologies when analyzing control samples at different concentration levels. Once again, satisfactory recoveries between 90 ± 1% and 100 ± 2% (for MEKC) and 84.0 ± 0.5% and 105 ± 1% (for

UVSD) were obtained, confirming the suitability of the selected methodologies to follow adsorption experiments of carbamazepine onto soils.

Table 6.3. Average recoveries (%) (± standard deviation) of carbamazepine in control samples obtained by MEKC and UVSD at two concentration levels (2 and 8 mg L^{-1}; 1 and 4 mg L^{-1}, respectively). Data are an average of triplicate batch experiments.

	MEKC		**UVSD**	
	2 mg L^{-1}	*8 mg L^{-1}*	*1 mg L^{-1}*	*4 mg L^{-1}*
SLU	96 ± 3	100 ± 2	93.0 ± 0.2	96.0 ± 0.2
COM	94.8 ± 0.7	99 ± 1	99 ± 1	105 ± 1
MIN	93 ± 2	90 ± 1	86.7 ± 0.6	84.0 ± 0.5

6.3.3 Adsorption experiments

6.3.3.1 UVSD analysis

The samples were diluted by a factor of two prior to UVSD analysis to avoid potential problems caused by the high concentrations of dissolved organic matter. In Figure 6.1a, examples of spectra of reference solutions (0.005 M $CaCl_2$, organic matter raw extract and carbamazepine) and a spectrum of a sample obtained by batch experiments with the soil SLU are shown. By observing the residuals plots of the carbamazepine coefficient in the linear combination it was possible to conclude that the obtained residuals were independent of the detection wavelength in all the studied soils (example in Figure 6.1b). This leads to the conclusion that the quantification of carbamazepine by UVSD was not affected by systematic errors and the wavelength range used to the UVSD analysis was adequately selected.

6.3.3.2 Comparison between MEKC and UVSD

Carbamazepine adsorption isotherms were obtained by plotting the amount of carbamazepine adsorbed per unit weight of soil (Q_e) as a function of the carbamazepine concentration that remained in solution (C_e) and by fitting the Freundlich equation (equation 6.2) to the results. The results obtained by MEKC and UVSD for the 3 selected soils are presented in Figure 6.2.

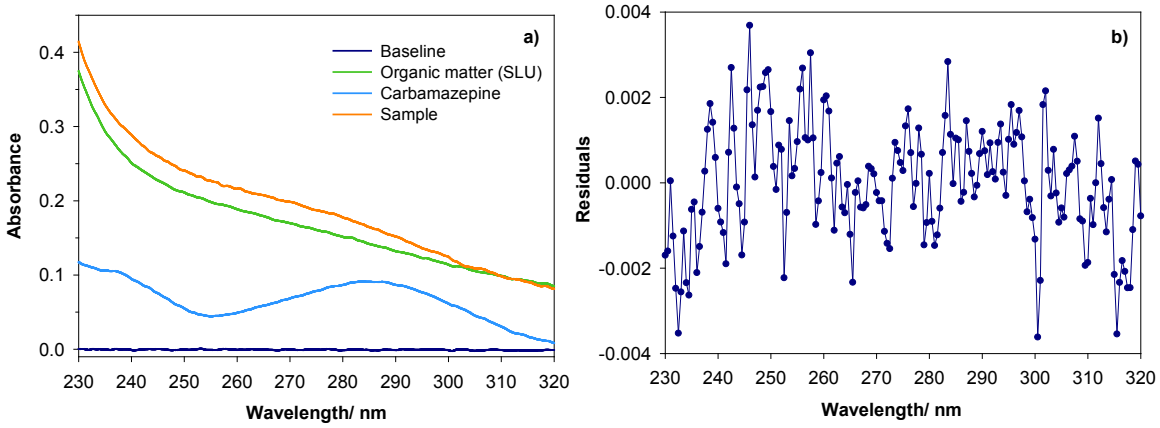

Figure 6.1. a) UV spectra of reference solutions for adsorption experiments with soil fertilized with sewage sludge (background, organic matter raw extract and carbamazepine) and total UV spectrum of a sample, in the wavelength range used to perform the deconvolution. b) Residuals of the carbamazepine coefficient as a function of the wavelength.

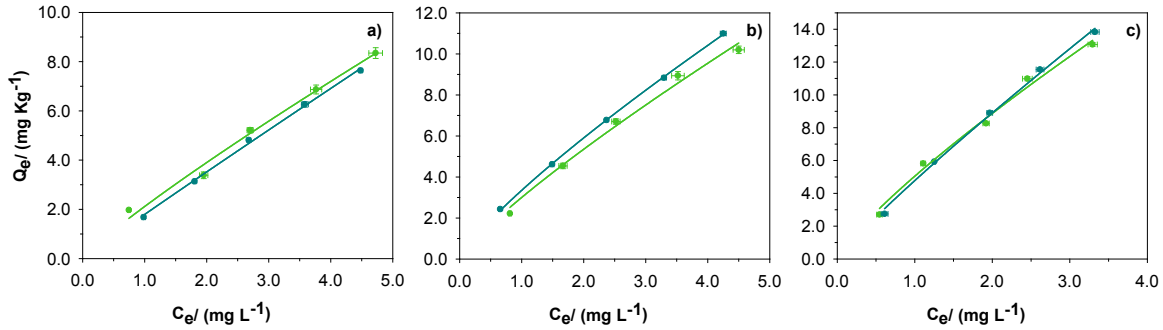

Figure 6.2. Comparison of adsorption isotherms obtained for carbamazepine with the soils a) MIN (mineral fertilization), b) SLU (sewage sludge fertilization) and c) COM (compost fertilization). Results obtained by MEKC and UVSD are shown in green and blue, respectively and are an average of triplicate batch experiments (± standard deviation).

In the Table 6.4, the Freundlich adsorption coefficient K_F, the degree of non-linearity N and the correlation coefficient r of the fitting to the Freundlich equation are shown. The Freundlich equation is an adequate model to describe the carbamazepine adsorption onto these soils, as the correlation coefficients range from 0.994 to 0.996 and from 0.999 to 1.000 for MEKC and UVSD, respectively. A two-tailed *t*-student test was performed to investigate significant differences between the results determined by both methods. The calculated *t* values are lower than the critical *t* value at 95% confidence level (with 4 degrees of freedom) for the two evaluated parameters and for all the soils. This test confirms that there are no statistical differences between the results

obtained by both methodologies, validating the UVSD method to follow the adsorption of carbamazepine onto soils.

Table 6.4. Freundlich adsorption coefficients (K_F) and degree of non-linearity (N) for the adsorption of carbamazepine onto agricultural soils subject to long-term fertilizations: sewage sludge (SLU), compost (COM) and mineral (MIN). Results are an average of triplicate adsorption batch experiments (± standard deviation). The two-tailed t-student test is also presented.

		MEKC	UVSD	t-student*
SLU	K_F /($mg^{1-N} L^N Kg^{-1}$)	3.0 ± 0.2	3.35 ± 0.04	2.68
	N	0.84 ± 0.06	0.82 ± 0.01	0.48
	r	0.996	1.000	-
COM	K_F /($mg^{1-N} L^N Kg^{-1}$)	5.0 ± 0.3	4.8 ± 0.2	1.32
	N	0.82 ± 0.07	0.90 ± 0.04	1.77
	r	0.994	0.999	-
MIN	K_F /($mg^{1-N} L^N Kg^{-1}$)	2.1 ± 0.2	1.79 ± 0.07	2.56
	N	0.88 ± 0.08	0.97 ± 0.03	1.58
	r	0.995	0.999	-

* For 4 degrees of freedom at 95% confidence level, the critical t value is 2.78 (two-tailed test).

6.3.4 Environmental relevance of the results

Carbamazepine adsorption presents a non-linear behavior (N ranging from 0.82 ± 0.07 to 0.88 ± 0.08 and from 0.82 ± 0.01 to 0.97 ± 0.03, as determined by MEKC and UVSD, respectively) which indicates a decrease on the tendency to adsorb onto soils with the increase of the initial concentration of carbamazepine in solution. Also, the results emphasize that the adsorption behavior of carbamazepine is clearly dependent on the soil organic matter content. Higher adsorption coefficients (see Table 6.4) were obtained for the soil with the highest content of organic matter (COM, total organic matter 9.4 ± 1.0%) and lower coefficients were obtained for the soil with the lowest content of organic matter (MIN, total organic matter 5.3 ± 0.3%). K_F values normalized to the soils' TOC (K_{FOC}) were also determined, using K_F coefficients obtained by MEKC (Table 6.5). The normalized K_F coefficient is independent of the total content of organic carbon and only depends on the organic matter specific characteristics (such as origin and genesis, which are also known to influence the sorption capacity of a soil). After normalization, very similar K_{FOC} were obtained, ranging from 151.5 to 181.2. The highest normalized adsorption coefficient was still observed for the COM soil, similarly to the non-normalized results; however, the MIN soil (the soil with lowest organic matter content) has a higher K_{FOC} than the SLU soil (Table 6.5),

indicating that the specific characteristics of the MIN soil organic matter are more favorable to the adsorption of carbamazepine than those of the SLU soil. Moreover, a concentration specific distribution coefficient (K_d^*), independent of the isotherm linearity, was determined and the results were also normalized to the soils' TOC (K_{oc}^*). These values are consistent with the conclusions taken for K_F and K_{FOC}. However, K_d^* and K_{oc}^* also allow a proper comparison with data from other studies where the linearity of the adsorption isotherm is different or the Freundlich fitting is not applied. Results are gathered in Table 6.5.

Table 6.5. Freundlich adsorption coefficient (K_F in $mg^{1-N}\ L^N\ Kg^{-1}$), concentration specific distribution coefficient (K_d^* in $L\ Kg^{-1}$, determined using an initial concentration of 6 $mg\ L^{-1}$) and respective values normalized to the total organic carbon content (K_{FOC} and K_{OC}^*). The presented parameters were calculated with data determined by MEKC.

	K_F	K_{FOC}	K_d^*	K_{OC}^*
SLU	3.0 ± 0.2	151.5	2.6 ± 0.2	130.7
COM	5.0 ± 0.3	181.2	4.4 ± 0.4	161.1
MIN	2.1 ± 0.2	169.4	1.9 ± 0.2	150.3

In the literature, there are three main studies about the sorption characteristics of carbamazepine onto soils, considering soils with different properties and organic matter contents (Williams et al., 2006; Chefetz et al., 2008; Yu et al., 2009). Yu et al. (2009) reported K_F coefficients between 0.10 ± 0.01 and 14.3 ± 0.01 $mg^{1-N}\ L^N\ Kg^{-1}$, depending on the soil under study and Chefetz et al. (2008) determined K_{OC} values from 116.3 to 176.0. On the whole, results from these two studies are in good agreement with this work, describing carbamazepine as a medium-low adsorption affinity pharmaceutical with a non-linear behavior. On the other hand, Williams et al. (2006) reported higher K_{OC} values (from 885 to 1250) and identified carbamazepine as a pharmaceutical that highly adsorbs onto soils and with low soil mobility. These differences should be indicative that the sorption behavior of carbamazepine exhibits a significant dependence of several factors such as soil composition, fertilization or irrigation conditions, which also make an accurate comparison of inter-study results harder.

Overall, the relatively low adsorption coefficients determined in this study, even considering the soil fertilized with compost, demonstrate that this pharmaceutical, apart from quickly adsorbing onto soils (very fast adsorption kinetics), is not extensively adsorbed. Hence, it is reasonable to expect that a significant percentage of carbamazepine present at the water/soil

interface remains in solution. It is also expectable that soils that frequently receive wastewater effluents and sludge constitute a source of carbamazepine to adjacent rivers and ground waters.

6.4 CONCLUSIONS

The adsorption behavior of carbamazepine onto agricultural soils was found to be dependent on soil fertilization (directly related to its organic matter content): higher organic matter content resulted in higher sorption capacity. The results highlight that carbamazepine is not extensively adsorbed onto soils particularly in the case of those subjected to mineral fertilization. Consequently, contaminated soils (due to the common use of effluents for crop irrigation and application of sludge) may be a potential source of carbamazepine into adjacent surface and ground waters by leaching. The results here presented underline the environmental risks involved in these applications of treated effluents and sludge, taking into account that the presence of carbamazepine in these matrices is generalized worldwide.

The medium-low distribution coefficients also indicate that, when in the water/soil interface, a significant amount of carbamazepine should remain in the aqueous phase which further validates the adequacy of this anti-epileptic to be used as a marker of anthropogenic pollution.

In what concerns the adopted UVSD method, this study shows that it constitutes a valid and accurate approach to follow sorption experiments onto soils. The use of this methodology, in comparison with the reference method used (MEKC), has the main advantage of being a simple and fast procedure, enabling the analysis of a large number of samples in a few hours and with low equipment requirements.

6.5 REFERENCES

Aboul-Kassim, T.A.T., Simoneit, B.R.T., **2001**. *Pollutant-Solid Phase Interaction - Mechanisms, Chemistry and Modelling*. Springer-Verlag, Berlin.

Carmosini, N., Lee, L.S., **2008**. Sorption and Degradation of Selected Pharmaceuticals in Soil and Manure. in: Aga, D.S. (Ed.). *Fate of Pharmaceuticals in the Environment and in Water Treatment Systems*. CRC Press, Boca Raton.

Chefetz, B., Mualem, T., Ben-Ari, J., **2008**. Sorption and mobility of pharmaceutical compounds in soil irrigated with reclaimed wastewater. *Chemosphere* 73, 1335-1343.

Coulomb, B., Richardson, Y., Brach-Papa, C., Boudenne, J.L., Theraulaz, F., **2006**. Rapid estimation of TOC in a marine urban sewage area by UV spectral deconvolution. *Int. J. Environ. An. Ch.* 86, 1079-1093.

Escalas, A., Droguet, M., Guadayol, J.M., Caixach, J., **2003**. Estimating DOC regime in a wastewater treatment plant by UV deconvolution. *Water Res.* 37, 2627-2635.

Gielen, G.J.H.P., Heuvel, M.R.v.d., Clinton, P.W., Greenfield, L.G., **2009**. Factors impacting on pharmaceutical leaching following sewage application to land. *Chemosphere* 74, 537-542.

Glassmeyer, S., Kolpin, D.W., Furlong, E.T., Focazio, M.T., **2008**. Environmental presence and persistence of pharmaceuticals: an overview. in: Aga, D.S. (Ed.). *Fate of Pharmaceuticals in the Environment and in Water Treatment Systems*. CRC Press, Taylor & Francis Books, Boca Raton, pp. 3-51.

Hassouna, M., Theraulaz, F., Massiani, C., **2007**. Direct estimation of nitrate, total and fractionated water extractable organic carbon (WEOC) in an agricultural soil using direct UV absorbance deconvolution. *Talanta* 71, 861-867.

J.N. Miller, Miller, J.C., **2005**. *Statistics and Chemometrics for Analytical Chemistry*, 5th ed. Prentice Hall, Dorset England.

Kibbey, T.C.G., Chen, L., Singhaputtangkul, N., Sabatini, D.A., **2009**. A UV-transparent passive concentrator/spectrum deconvolution method for simultaneous detection of endocrine disrupting chemicals (EDCs) and related contaminants in natural waters. *Chemosphere* 76, 1249-1257.

Levine, A.D., Asano, T., **2004**. Recovering sustainable water from wastewater. *Environ. Sci. Technol.* 38, 201A-208A.

Lima, D.L.D., Santos, S.M., Scherer, H.W., Schneider, R.J., Duarte, A.C., Santos, E.B.H., Esteves, V.I., **2009**. Effects of organic and inorganic amendments on soil organic matter properties. *Geoderma* 150, 38-45.

Lima, D.L.D., Silva, C.P., Erny, G.L., Esteves, V.I., **2010**. Comparison between MEKC and UV spectral deconvolution to follow sorption experiment in soil. *Talanta* 81, 1489-1493.

Limousin, G., Gaudet, J.P., Charlet, L., Szenknect, S., Barthès, V., Krimissa, M., **2007**. Sorption isotherms: A review on physical bases, modeling and measurement. *Appl. Geochem*. 22, 249-275.

Nam, P.H., Alejandra, B., Frédéric, H., Didier, B., Olivier, S., Pauss, A., **2008**. A new quantitative and low-cost determination method of nitrate in vegetables, based on deconvolution of UV spectra. *Talanta* 76, 936-940.

OECD, **2000**. *Adsorption – Desorption using a Batch Equilibrium Method*. Guideline for the Testing of Chemicals nº 106, Paris.

Pedersen, J.A., Soliman, M., Suffet, I.H., **2005**. Human pharmaceuticals, hormones, and personal care product ingredients in runoff from agricultural fields irrigated with treated wastewater. *J. Agr. Food Chem*. 53, 1625-1632.

Thomas, O., Theraulaz, F., Domeizel, M., Massiani, C., **1993**. UV spectral deconvolution – a valuable tool for waste-water quality determination. *Environ. Technol.* 14, 1187-1192.

Williams, C.F., Williams, C.E., Adamsen, E.J., **2006**. Sorption-desorption of carbamazepine from irrigated soils. *J. Environ. Qual.* 35, 1779-1783.

Wu, C., Spongberg, A.L., Witter, J.D., Fang, M., Czajkowski, K.P., Ames, A., **2010**. Dissipation and leaching potential of selected pharmaceutically active compounds in soils amended with biosolids. *Arch. Environ. Con. Tox.* 59, 343-351.

Yu, L., Fink, G., Wintgens, T., Melin, T., Ternes, T.A., **2009**. Sorption behavior of potential organic wastewater indicators with soils. *Water Res*. 43, 951-960.

CHAPTER 7

Concluding remarks

CONTENTS

7.1 CONCLUSIONS

The occurrence of psychiatric pharmaceuticals in the environment has recently raised a widespread concern about the serious effects that their presence might have in the ecosystems. As a part of the global effort that, in the last few years, is being made to improve the understanding of this multidisciplinary issue, the work presented in this thesis is intended to positively contribute to the clarification of some important processes that affect the environmental occurrence and fate of psychiatric pharmaceuticals.

Overall, and in what concerns the anti-epileptic pharmaceutical carbamazepine, the evidence presented in this thesis allows to support the hypothesis which considers carbamazepine as an adequate marker of anthropogenic pollution. Carbamazepine has shown to be particularly resistant to direct photodegradation under environmentally relevant conditions and, in water/soil interfaces it remains in the aqueous phase in significant amounts. The described results, in conjunction with the global high levels of consumption and its resistance to WWTP degradation processes (well document in the literature and confirmed in Chapter 5), make this pharmaceutical a good target compound to the identification of relevant focus of pollution, caused by domestic and urban wastewaters. In line with this assessment, this work was also concerned with the development of a fast and reliable method to the determination of such an important pollutant in many types of real aqueous matrices. As a result, an enzyme-linked immunosorbent assay was developed and optimized, being an important tool to perform future large-scale screenings.

This thesis also presents results concerning the direct and indirect photodegradation of benzodiazepines under environmentally relevant conditions. This was the first work to give insights into the relevance of photo processes in the persistence of this group of pharmaceuticals. The reported data revealed that pharmaceuticals such as diazepam, oxazepam and alprazolam are not significantly eliminated from aquatic environments by photodegradation, at a reasonable time scale. On the contrary, the benzodiazepine lorazepam was quickly eliminated under the same conditions. Environmental half-lives of diazepam, oxazepam and alprazolam seem to be higher than the ones observed for carbamazepine (which is already described in literature as an extremely resistant compound). This fact is a clear indication that studying benzodiazepines under environmental conditions should be a priority issue in future research. Furthermore, this study also elucidated that distinct fractions of dissolved organic matter influence the photodegradation processes of these compounds differently.

One of the main focuses of this research work was the identification of the direct photodegradation products of the five selected compounds. The majority of the identified photoproducts is documented here for the first time. This identification is crucial for future investigations as it will allow evaluating whether these photoproducts occur in the environment in significant quantities (in relation to the parent compound) and to assess potential toxicological problems that may be derived from their presence.

7.2 FUTURE WORK

As was emphasized along this document, the existing knowledge about the phenomena that dictate the fate and persistence of psychiatric pharmaceuticals is still limited. Thus, as a result of the novelty and complexity of the addressed topics (and also inherently to the scientific research process) the work here presented not only provides answers but also raises a number of pertinent questions.

Concerning photodegradation processes, the reasons behind the observed influence of environmentally relevant parameters such as dissolved oxygen and dissolved organic matter is still lacking a consistent and mechanistic elucidation. In this context, further work should be performed in order to clarify the chemical species involved, for instance, in the enhancement of carbamazepine´s photodegradation in the absence of oxygen and also in the enhancement of benzodiazepines' photodegradation in the presence of some fractions of humic substances and in a larger range of concentrations. Moreover, and in order to provide progressively more environmentally relevant data, it should be interesting to perform photodegradation experiments using real aqueous matrices from different proveniences and with different well-defined characteristics. These studies would allow not only to have an even more realistic idea about the resistance of these pharmaceuticals to photodegradation in real environments, but also to identify the individual components responsible for enhancing/inhibitory effects.

In order to complement the performed photodegradation studies of benzodiazepines, and similarly to carbamazepine, the behavior of these psychiatric pharmaceuticals in water/soil interfaces should be investigated. The preference of these compounds to remain in the aqueous or in the solid phase will largely influence the relevance of the described photodegradation processes and will positively contribute to the definition of the most relevant issues to be addressed in future work.